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Control of p27 Localization and Degradation by the PI3 Kinase Akt/PKB pathway in MCF-7 Breast Cancer Cells

Marissa J. Bradbury
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Marissa J. Bradbury entitled "Control of p27 Localization and Degradation by the PI3 Kinase Akt/PKB pathway in MCF-7 Breast Cancer Cells." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Jay Wimalasena, Major Professor

We have read this dissertation and recommend its acceptance:

Hwa-Chain Robert Wang, Sundar Venkatachalam

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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A Thesis Presented for the Master of Science Degree
The University of Tennessee, Knoxville

Marissa J. Bradbury
December 2004

DEDICATION

This thesis is dedicated to my parents

Mr. David Bradbury

Mrs. Karen A. Bradbury,

And my sisters,

Ms. Jessica Bradbury

Ms. Alicia Bradbury

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I am grateful to many people for their help and advice during my studies at the University of Tennessee. I am most grateful to my major professor, Dr. Jay Wimalasena, for the opportunity to work in his laboratory and for his advice and guidance. I am also thankful to my committee members, Dr. HR Wang and Dr. Sundar Venkatachalam for their advice and support. I am especially grateful Dr. Romaine Ingrid Fernando, for teaching and guiding me throughout my studies. I would like to thank Don Henley for his advice and organizational skill, and Steve Foster, for his in depth knowledge of the subject matter, awesome teaching ability, assistance, advice and ideas.

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ABSTRACT

Breast cancer is caused by a variety of environmental and genetic factors that influence cell growth and survival. Changes in the level or function of cell cycle regulatory proteins are often associated with breast cancer. Low expression of the cell cycle inhibitor, $p27^{Kip1}$, is associated with aggressive breast tumors. Clinical observations have inspired studies to analyze p27 as a potential target for signal transduction pathways in cancer cells. The stability of p27 determines it's ability to regulate the cell cycle, and changes in transcription and translation of p27 are less influential. The regulation of the cellular localization of p27 by signal transduction pathways has become a focus of recent research. This study attempts to clarify how Akt regulates p27 localization and stability, and what implications this data may have on tumor growth and metastasis. Cells were transfected with MryAkt plasmid to artificially activate Akt. Immunoflorescence and cell fractionation results verified that MryAkt causes cytoplasmic retention of p27 in MCF-7 breast cancer cells. Labeling experiments with ^{35}S methionine demonstrated that MryAkt causes nascent p27 to accumulate in the cytoplasm .Metabolic pulse chase labeling and cyclohexamide experiments also determined that p27 was stabilized by MryAkt. Degradation in the nuclear and cytoplasmic compartments was unaffected by Akt, which supports the hypothesis that MryAkt effects only p27 localization, and the stabilization of p27 by Akt is a result of its cytoplasmic retention. Lastly, the mitogen estradiol has similar effects on p27 as MryAkt, proving that estrogen can influence p27 through the Akt pathway.

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The Cell Cycle

The eukaryotic cell cycle is regulated by cyclin dependent kinases (Cdks). Cdks are activated when proteins called cyclins bind to them and form complexes. In each cell cycle, cyclins are regulated by a series of synthesis and degradative possesses, and changes in cyclin levels result in the assembly and activation of the cyclin /Cdk complexes that trigger the cell cycle events. G_1 phase of the cell cycle is regulated by cyclin D and Cdk 4/6, G_1/S is initiated by cyclin E/Cdk2, S phase is regulated by cyclin A/Cdk2, and m phase or mitosis is controlled by cyclin B/Cdk1 (Cdc2). Cdks are inactive when cyclins are not bound. Cdks have their active site blocked by the t-loop region of the protein. When cyclins bind this causes the t-loop to move away from the active site, then a cyclin activating kinase (CAK) phosphorylates the complex at a threonine residue in the t-loop. This fully activates the enzyme complex by changing the shape of the t-loop an improving the ability of the enzyme to bind substrate. A cell cycle inhibitor protein (CKI), $p27^{kip1}$ (p27) or $p21^{cip\ 1}$ (p21), can bind to the cyclin- Cdk complex and distort the active site of the Cdk. Also they can insert into the ATP binding site. p27 and p21 form trimeric complexes with cyclin E or A Cdk2 complexes to inactivate their enzyme activity. Previously it was thought that at physiological concentrations, p21 and p27 promote formation of active D cyclin-Cdk4/6 complexes but at higher concentrations they inhibit Cdk4/6 activity. Now recent studies have shown that cyclin D complexes form independently of p21 and p27 (1) (2). The function of cyclinD/Cdk4/6 is to sequester p27 away from cyclin E/Cdk2, then cyclin E/Cdk2 and

cyclinA/Cdk2 can be activated and initiate G_1/S phase transition. Also Cdk4 is responsible for phosphorylating Retinoblastoma protein (explained below) (3).

INK4 Proteins

Members of the INK4 protein family can inhibit Cdk4/6, prevent them from binding to cyclin D, which prevents the G1/S transition of the cell cycle. This family consists of P16INK4a, P15INK4b, P18INK4c and p19INK4d which share a common structural motif, the ankyrin repeats, that have pairs of anti-parallel α-helices stacked side by side and connected by a series of intervening hairpin motifs (4). These domains of the INK4 proteins are responsible for binding to the non-catalytic side of Cdk4 and Cdk6 (opposite to the cyclin D binding site) and this binding induces and allosteric change in the Cdks which alters the binding of D type cyclins and reduces their affinity for ATP. Furthermore, this prevention of cyclin D complex formation redistributes the inhibitors p27 and p21 to cyclin E/Cdk2 complexes, which further inhibits G_1/S phase transition.

The E2F Family and Retinoblastoma Protein

E2F and Retinoblastoma protein (Rb) are important contributors to the G_1/S phase transition of the cell cycle. pRb is a tumor suppressor gene that is absent or mutated in at least one- third of human tumors, including familial inactivating tumors that are associated with the pediatric eye tumor retinoblastoma. Rb is a member of a family of proteins called the pocket proteins that also includes p107 and p130 (5). Rb binds to the transcription factor E2F in the large pocket region (amino acids 395-876) and suppresses E2F activation. The small pocket region of Rb (379-792), binds to the LXCXE region of

E2F, and viral oncoproteins can also bind to this region and interfere with Rb/E2F binding (6). Figure 1 shows an illustration of Rb and E2F-1 function in the cell cycle.

The E2F protein belongs to a family of similar proteins divided into groups based on sequence homology: E2F-1, 2 and 3 in one subgroup and E2F-4 and 5 in the second group. A third subgroup exists containing E2F-6, which suppresses transcription independent of pocket protein binding. This protein has almost no homology outside the DNA binding and dimerization, and has truncated C and N terminal groups. Group one E2F proteins possess an N-terminal cyclinA/Cdk2 binding domain and a canonical basic nuclear localization signal (NLS). E2F-1, 2&3 preferentially associate with Rb, while E2F-4&5 associates with p107 and p130 (6).

E2F proteins are transcription factors that exist as heterodimers with one member of the E2F protein family and one member of the DRTF1 (DP) family. The six members of the E2F family can form different combinations of heterodimers with the two members of the DP family. E2F was identified as a cellular factor required for the adenovirus early region 1A transforming protein to mediate transcriptional activation of the viral E2A promoter. DP was discovered because its DNA binding activity decreased during differentiation of F9 embryonic carcinoma stem cells. Collectively, DP and E2F were shown to have similar DNA binding sequences, and now the activity of this heterodimer is referred to as simply E2F (7).

G_1/S Transition of The Cell Cycle

In G_1 phase of the cell cycle, cyclin DCcdk4/6 complexes initiate Rb phosphorylation. When cyclin E/Cdk2 becomes active at the end of G_1, it phosphorylates

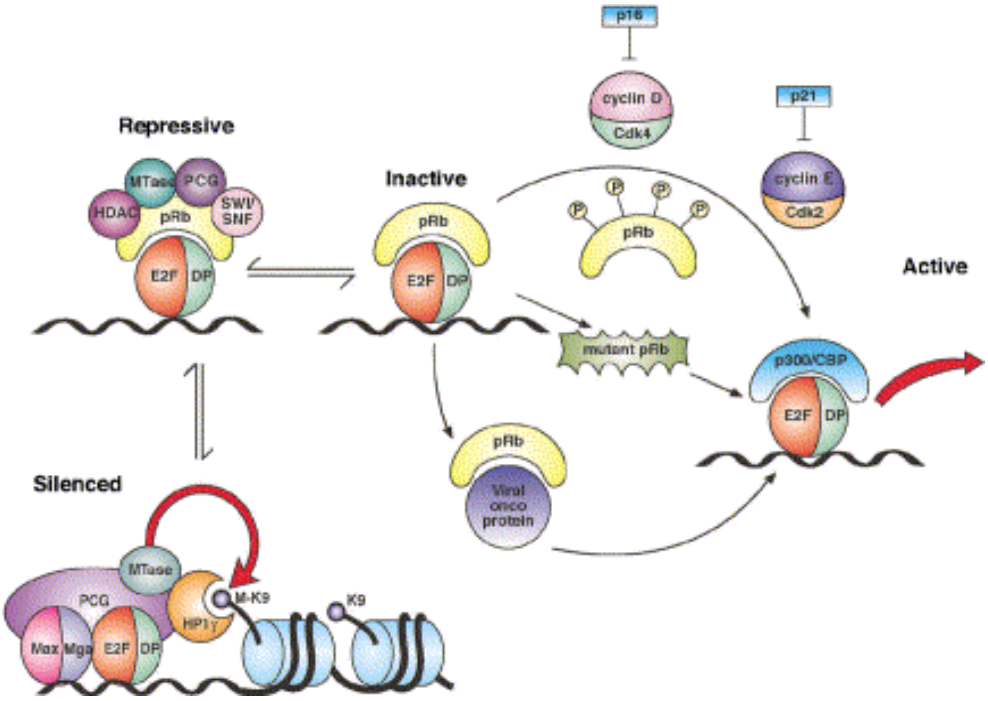

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Figure 1: Regulation of E2F activity

Rb suppresses E2F activity until it is phosphorylated by the cyclin D/cdk4 complex. Free E2F becomes transcriptionally active and interacts with the cofactors p300/CBP. The pRB/E2F complex represses transcription by recruiting proteins HDAC, MTase, PCF and SWI/SNF that alter the chromatin environment to favor transcriptional inactivity.

Rb on additional sites. The hyperphosphorylation of Rb in late G_1 disrupts its association with E2F and allows this protein to initiate the transcription of genes necessary for DNA synthesis. Once free from inhibition by Rb, E2F drives cells into S phase as it regulates transcription factors that initiate the G_1 to S transition. Repression by the Rb-E2F complex is required to force G_1 arrest by anti-proliferative signals. Subsequent activation of Cyclin A and B Cdks maintains Rb in the hyperphosphorylated form until the cells exit mitosis and Rb is returned to it's hypophosphorylated state in the next G_1 (8). A representation of the cell cycle and important cell cycle regulators and their inhibitors is shown in figure 2.

Cdc2 and Cdk2 Regulation

Activation of Cdk2 is the key regulatory event at the G_1/S phase transition. In addition to the CKIs, Cdk2 is also regulated and phosphorylated on threonine 160 by a heterotrimer consisting of Cdk7, cyclin H and Mat1 kinase (9). Another kinase, Wee1, phosphorylates Cdk2 on tyrosine 15 and deactivates it (10), (11). The phosphatase, Cdc25A, dephosphorylates tyrosine 15 and restores Cdk2 activity (12), (13).

In early G_2, Cdc2 becomes the predominant Cdk and this continues through M phase. Cdc2 is also deactivated by phosphorylation on tyrosine 15 by Wee1 and threonine 14 by a membrane associated kinase Myt1. Myt1 kinase inhibits Cdc2 both by its inhibitory phosphorylation and by sequestering Cdc2 at the cell membrane away from the nuclear compartment. Cdc25C dephosphorylates Myt1 phosphorylated Cdc2 and restores its activity (11-14).

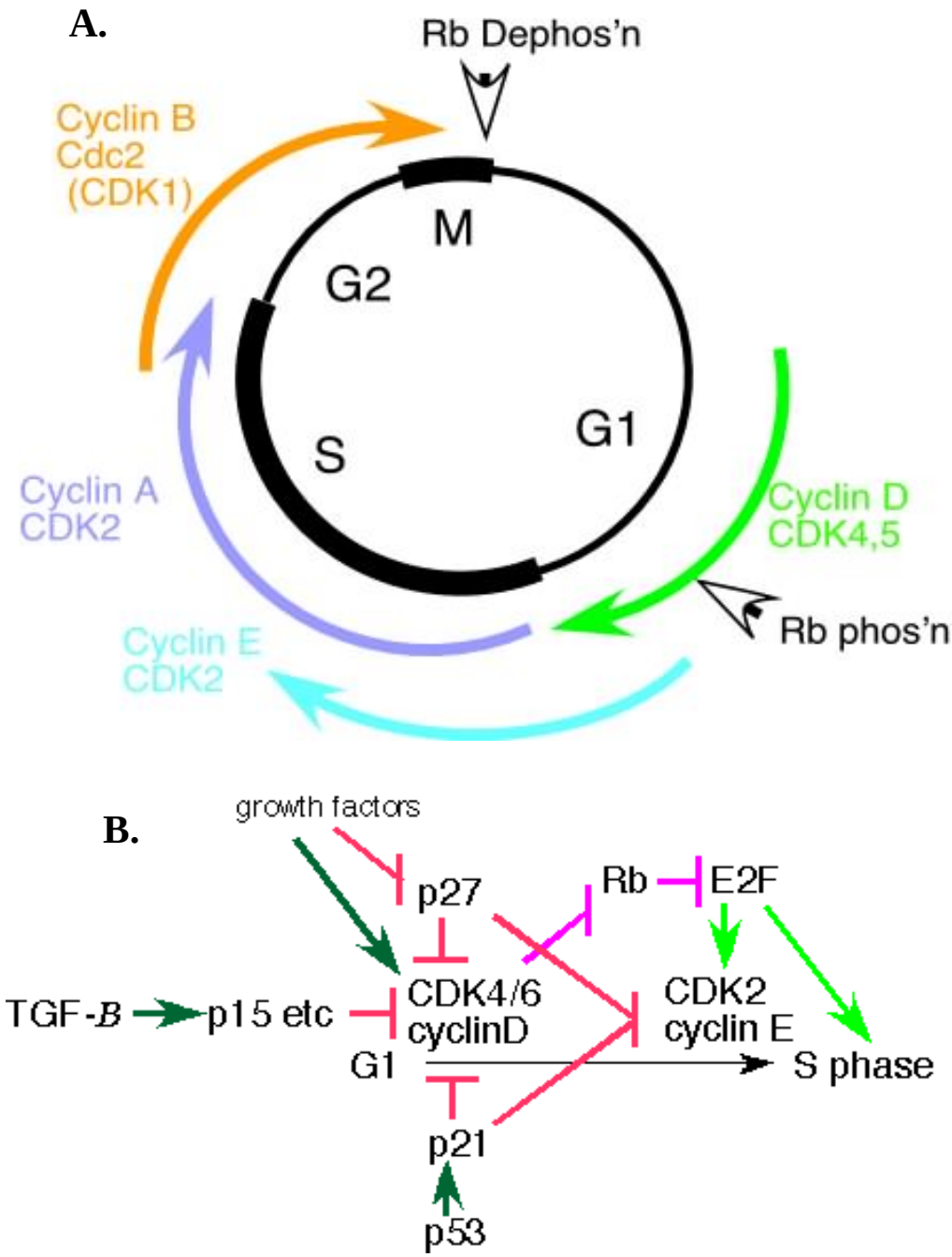


Figure 2: The mammalian cell cycle. (A) Arrows indicate the relative increase of the cyclin levels at different phases of the cell cycle. G1=gap 1, S=synthesis, G2=gap2, m=mitosis, CDK=cyclin dependent kinase, Rb=retinoblastoma protein. (B) Cell cycle proteins and their inhibitors. TGF-B=tumor growth factor beta. Arrows indicate activation, bars indicate inhibition.

p27 and Cancer

The cell cycle inhibitor, p27, has become an important focus of study in many types of cancer research. p27 could be and important protein target for cancer therapy. This is evident because the amount of p27 protein has been shown to be a possible prognostic marker in human neoplasms such as breast, lung, esophageal, and colorectal carcinomas. Low expression or increased degradation of p27 protein occurs in many human tumors, and this strongly correlates with tumor progression and poor prognosis (8). p27 knockout mice display gigantism with multi-organ hyperplasia, suggesting that p27 limits the replicative capacity of differentiating cells. Also p27-/- mice develop pituitary tumors, and have high susceptibility to carcinogen and radiation induced tumorigenesis. The mammary glands of p27 (-/-) mice are underdeveloped compared to wild type, but the mammary epithelium of p27 (+/-) mice is hyperproliferative and susceptible to oncogene induced tumorigenesis (14), (15). Much of p27 in cells is controlled post transcriptionally, i.e. p27 protein levels are controlled by ubiquitin-mediated proteolyses at the G1/S borders, although p27 can be controlled transcriptionally as well.

p27 Degradation and The SCF^{SKP2} Complex

The ubiquitination and subsequent degradation of nuclear p27 is carried out by a multi enzyme complex consisting of E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme, and E3 ubiquitin ligase (16), (17). This complex consists of adaptor molecules that ligate proteins to chains of a small peptide called ubiquitin, which allows the protein to be recognized and degraded by the proteosome (18). This mechanism is a

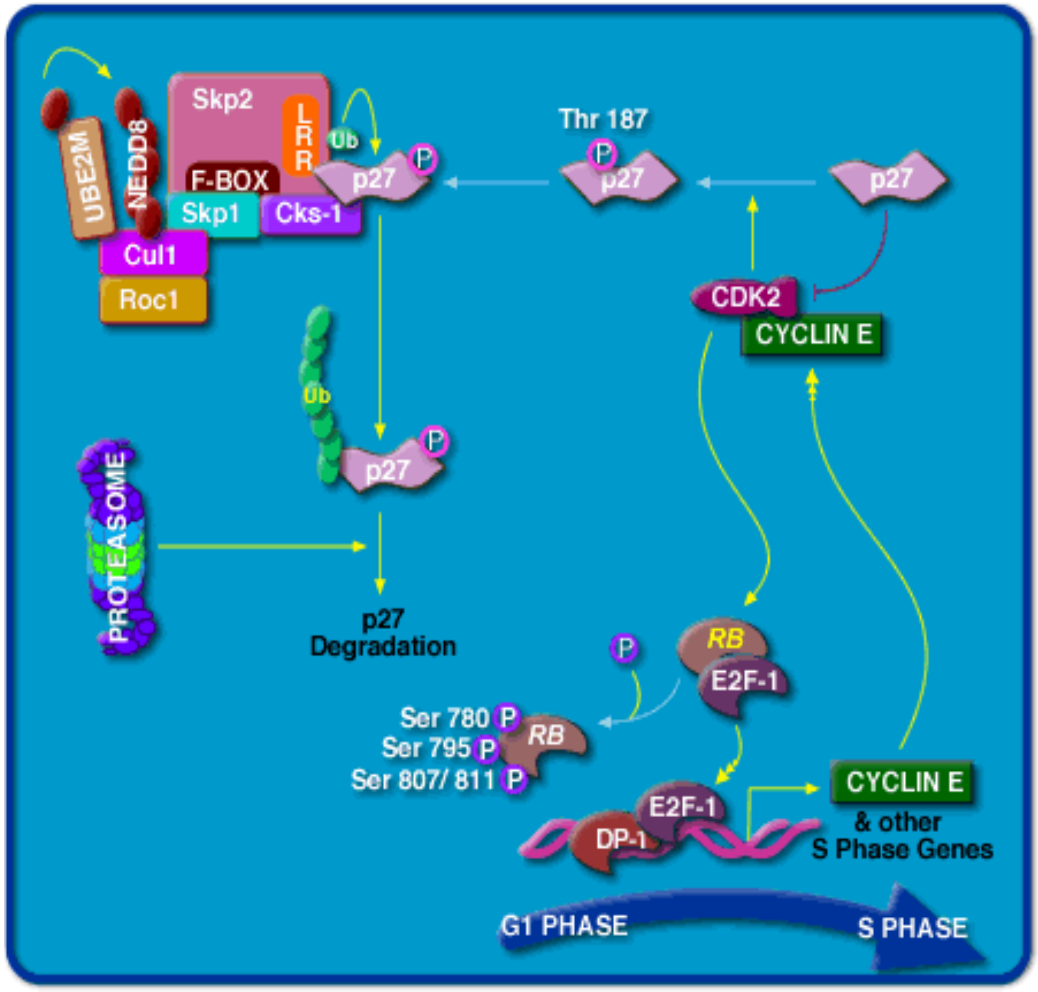
Skp2 protein dependent cyclin E/Cdk2 mediated degradation pathway (19). Ubiquitin dependent proteolysis of p27 is regulated by phosphorylation on threonine 187 by cyclin E/Cdk2 in late G_1. Phosphorylation on threonine 187 allows recognition of p27 by the SCF type E3 ligase complex (20). Polyubiquitinated p27 is then targeted to the 26s proteosome and degraded. The complex of proteins required for p27 degradation called the SCF^{SKP2} complex, contains Skp1, Skp2, Cul1, Cks1, and Rbx/Roc1 and is responsible for recognizing phosphorylated p27 and catalyzing ubiquitination (21), (22) . Part of the SCF complex, the protein Skp2, was originally identified as a protein associated with S phase promoting kinase Cdk2/cyclin A, and Skp2 activation is required for S phase entry (23). Skp2 is a member of a diverse family of proteins that share common sequence motif called the F box. Skp2 associates with peptides derived from the extreme carboxyl terminus of p27, and full-length p27. This association is responsible for recruiting p27 to the SCF complex. Skp1 directly binds to the F-Box motif and mediates assembly of the SCF^{SKP2} ubiquitin ligase complex (24). The center of the complex is Cullin (Cul1) which interacts with all three subunits and serves as a scaffold. Rbx/Roc1 binds to Cul1 and acts as an essential component of the E3 ubiquitin ligase (25). Rbx /Roc1 recruits the E2 enzyme, CDC34/UBC3, to the complex. The E2 and E1 enzymes transfer ubiquitin to the substrate (p27), and mark it for degradation (18). Also the complex requires an accessory protein called Cks-1 that binds Skp2. The two amino acid residues in Cks-1 that are required for interaction with Skp2 and ubiquitination of p27 have been identified (26). The paper by Wang et al shows that Cks-

1 binds to the carboxyl terminal region of Skp2 and that the amino acid Asp331 is essential for binding (27). An illustration of this complex and cell cycle regulators is shown in figure 3.

Studies have shown that p27 phosphorylated on serine 10 is more stable than the unphosphorylatable S10A mutant protein. p27 is phosphorylated on serine 10 by the His kinase (28). Phosphorylation on serine 10 accounts for about 70% of the total phosphorylation of p27 and is increased in G_0/G_1 phase of the cell cycle (29). In Skp2 -\- lymphocytes, p27 has been shown to behave similarly to p27 in wild type cells at the G_0/G_1 transition of the cell cycle, while proteolysis at the S/G_2 phases is impaired (30). This suggests that p27 also degrades by a Skp2 independent process in G_0/G_1 (31). Current evidence suggests that in fact p27 is degraded by a Skp2 independent ubiquitination pathway in the cytoplasm, and a Skp2 dependent ubiquitination pathway in the nucleus (30).

p27 and The MCF-7 Cell Cycle

Translational controls, and proteolysis in MCF-7 breast cancer cells and other cell types regulate p27 levels. p27 protein levels are decreased as cells progress from G_1 to S phase (32). p27 levels are elevated in G_0 phase of the MCF-7 cell cycle. In early G_1 after mitogen stimulation, cyclin D/cdk4 complex sequesters p27. In mid G_1 (8-10 hours), the levels of p27 begin to decline prior to an increase in Skp2 (3). This is thought to be due to nonspecific degradation in the cytoplasm, because p27 is exported to the cytoplasm by phosphorylation on serine 10 (and subsequent mitogen stimulation) and this process is independent of Skp2. In late G1, the SCF^{SKP2} ubiquitin ligase complex



Adopted from http:www.med.nyu.edu/cgi-bin

Figure 3. The SCF ^{Skp2} degradation complex
p27 is phosphorylated on threonine 187 by cdk2/cyclin E at G_1/S. p27 is then ubiquitinated by this complex then targeted to the 26S proteosome and degraded.

further degrades nuclear p27 (12-18 hours) (33). After S phase (12 hrs) the level of p27 begins to increase.

Estrogen and MCF-7 Cells

p27 protein levels are regulated by 17β estradiol (estrogen or E_2) in many cell types. Estrogen is a potent mitogen for estrogen receptor positive MCF-7 Cells. Estrogen stimulates proliferation by increasing synthesis of cyclin D and Skp2 proteins, while decreasing p27 protein levels. Also estrogen increases the activity of cyclin D/Cdk 4 complexes, which sequester p27 thereby increasing Cdk2 kinase activity (3). The classical steroidal action of estrogen is via two genetically different nuclear receptors, nERα and nERβ (34). These receptors have divergent functions and cellular actions. 17β estradiol, which has a higher affinity for ERα, transfuses the membrane and binds the ER in the cytoplasm. The receptors undergo conformational changes, form heterodimers or homodimers, and translocate to the nucleus where they activate transcription factors and initiate the synthesis of proteins. ERα seems to be a more potent activator than ERβ, and when both receptors are expressed ERβ can decrease ERα activity (34). The same ER ligand complex is not recognized similarly in all cells, which accounts for the diversity of ER function. This is partly due to the two classes of co-regulators of ERs. Co-activators nucleate protein complexes at gene promoters to enhance transcription. Co-repressors recruit proteins that lead to the suppression of transcriptional activation (35). Activated ERs can initiate transcription by binding to estrogen response elements (EREs) within promoters. Also they can indirectly increase transcription through the activation of transcription factors (TF) like AP1, or SP1, or they can suppress transcription by

inhibiting TF like Nf-kB. This genomic action of estrogen via the nuclear receptors is characterized by a latency of onset of 2-8 hours (36).

Now estrogen is known to have non-genomic effects, which are described by a short response time, insensitivity to inhibitors of transcription and translation, and different properties than nuclear receptors i.e. different antagonists. These rapid effects appear to originate from plasma membrane receptors located in the caveolae and to date, a membrane ER has been partially characterized. Theoretically, through binding to membrane receptors, this steroid can activate tyrosine kinase receptors like EGFR and G protein coupled receptors (38), which activate signal pathways including the Ras/Raf/Mek/Erk (39), (40) pathway and the Ras PI3 K/Akt pathway (39) (41).

p27 Localization

It has recently been noted that p27 cytoplasmic and nuclear localization may be important in MCF-7 breast cancer cells and other cell types. The transport of proteins from the nucleus to the cytoplasm is mediated by a nuclear export signal (NES), which consists of a conserved set of leucine residues. Mutations of these residues disrupt this export process. The protein, Crm-1 (part of the Importin B related nuclear transport receptors) associates with the NES and mediates export of proteins including p27 (42). When p27 is phosphorylated on this site and mitogen is available, Crm-1 dependent export occurs and p27 is transported to the cytoplasm (33). Leptomycin B (LMB) can inhibit this export process by binding to Crm-1 and preventing it from interacting with the NES of substrate proteins (42).

p27 interaction with the nuclear pore protein NUP50/NPAP60 may have a role in nuclear import (43) (44). A nuclear localization signal (NLS) on p27 has been identified and may have other mediators (45), (46). The phosphorylated p27 interacts with a protein called Jab1 and cotransfections with these two genes showed increased proteolysis of p27 in certain cell types. The observation that LMB inhibited Jab-1 mediated export suggests that a CRM-1 dependent nuclear export mechanism influences degradation of p27 (47). CRM-1 and the GTPase Ran regulate nuclear import and export. In the nucleus, Ran-bound GDP exchanges for ran GTP (by the guanine exchange factor protein (GEF) RCC1) and binds export cargo destined for the cytoplasm. In the cytoplasm, Ran GTP dissociates the complex (48). In the paper by Connor et al, the nuclear export of p27 is regulated by Crm-1/Ran GTP binding and is time, temperature and energy dependent and a NES is required (49). Lastly, a recent study has demonstrated that Crm-1 dependent export of p27 requires its phosphorylation at serine 10 and stimulation of the Ras/Raf/Mek/Erk pathway by mitogens (33).

PI3 Kinase and Akt/PKB

p27 localization is influenced by the PI3 Kinase Akt pathway in certain cell types (52), (53), (54). There are three PKB/Akt genes in metazoans which yield the protein products PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3. The three genes are regulated similarly and have similar structures; they contain an amino-terminal pleckstrin homology domain and require phosphorylation for activation. Akt belongs to the ACG family of protein kinases (protein kinase A, protein kinase C, and protein kinase G). This group requires phosphorylation of a flexible peptide t loop near the catalytic core of the

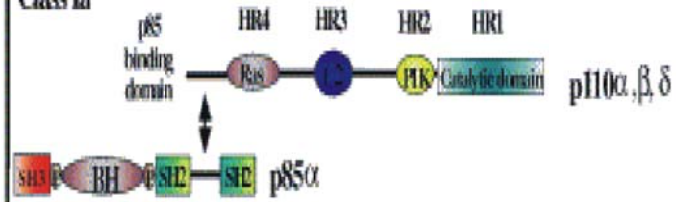
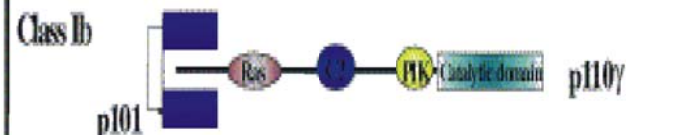
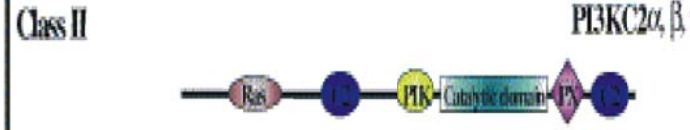
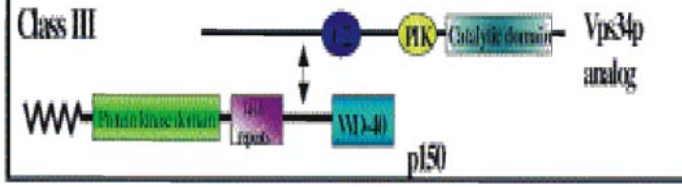
kinase domain for activation (50). Akt is activated by PI3 kinase. PI3 kinase is a heterodimeric complex consisting of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. There are eight mammalian PI3 kinases and they are categorized into three classes (I, II, and III) (51). A summary of these classes is shown in table one.

PI3 kinase is activated by receptor tyrosine kinases or G protein coupled receptors. When localized to the membrane via its regulatory 85 kDA subunit, (an SH2 domain containing subunit) it associates with tyrosine phosphorylated receptors or adaptors. Then the catalytic subunit is responsible for PI3 kinase catalyzing the phosphorylation of inositol phospholipids at the three position of the inositol ring to generate the lipids phosphatidylinositol 3, 4 bisphosphate PI (3,4) p2 and phosphatidlylinositol 3,4,5 triphosphate. PI (3,4,5,) p3. As the levels of these lipids rise, pleckstrin homology domain containing proteins like AKT and PDK1 are recruited to the membrane. PDK1 phosphorylates and activates AKT by phosphorylating it on threonine 308, which causes autophosphorylation of serine 473 (or phosphorylation by another kinase PDK2 but this is still being debated). PI3K is inactivated by phosphatases like PTEN (phosphatase and tensin homologue deleted from chromosome ten) that removes the phosphate from the three position on the inositol ring (50). Some downstream targets of PI3 kinase are shown in figure 4.

p27 Localization and Akt

Recently studies have demonstrated that p27 localization is important in cell cycle arrest in cancer cells. p27 is mislocalized to the cytoplasm in ovarian, breast and thyroid tumors (52). Recently it has been shown that AKT mediates cell cycle progression by

Table 1: Summary of the Classes and Function of PI3 Kinase

	Catalytic	Regulatory	Regulated by	Substrates *in vitro*	Substrates *in vivo*	Tissue Distribution
Class Ia 	p110α,β,δ	p85α,p85β p55γ P50α,p55α	tyrosine kinases Ras Gβγ for 110β	PtdIns PtdIns(4)P PtdIns(4,5)P_2	PtdIns(4,5)P_2	α,β-ubiquitous δ-leukocytes
Class Ib 	p110γ	p101	Gβγ Ras			myeloid
Class II 	PI3KC2-α,β,γ	?clathrin (α)	tyrosine kinases cheomokines integrins (not Ras)	PtdIns PtdIns(4)P	PtdIns(4)P	α,β-widespread (α-CCV,TGN) γ-hepatic (JNR, plasma/nuclear membranes)
Class III 	Vps34p analog	p150	?constitutive	PtdIns	PtdIns	

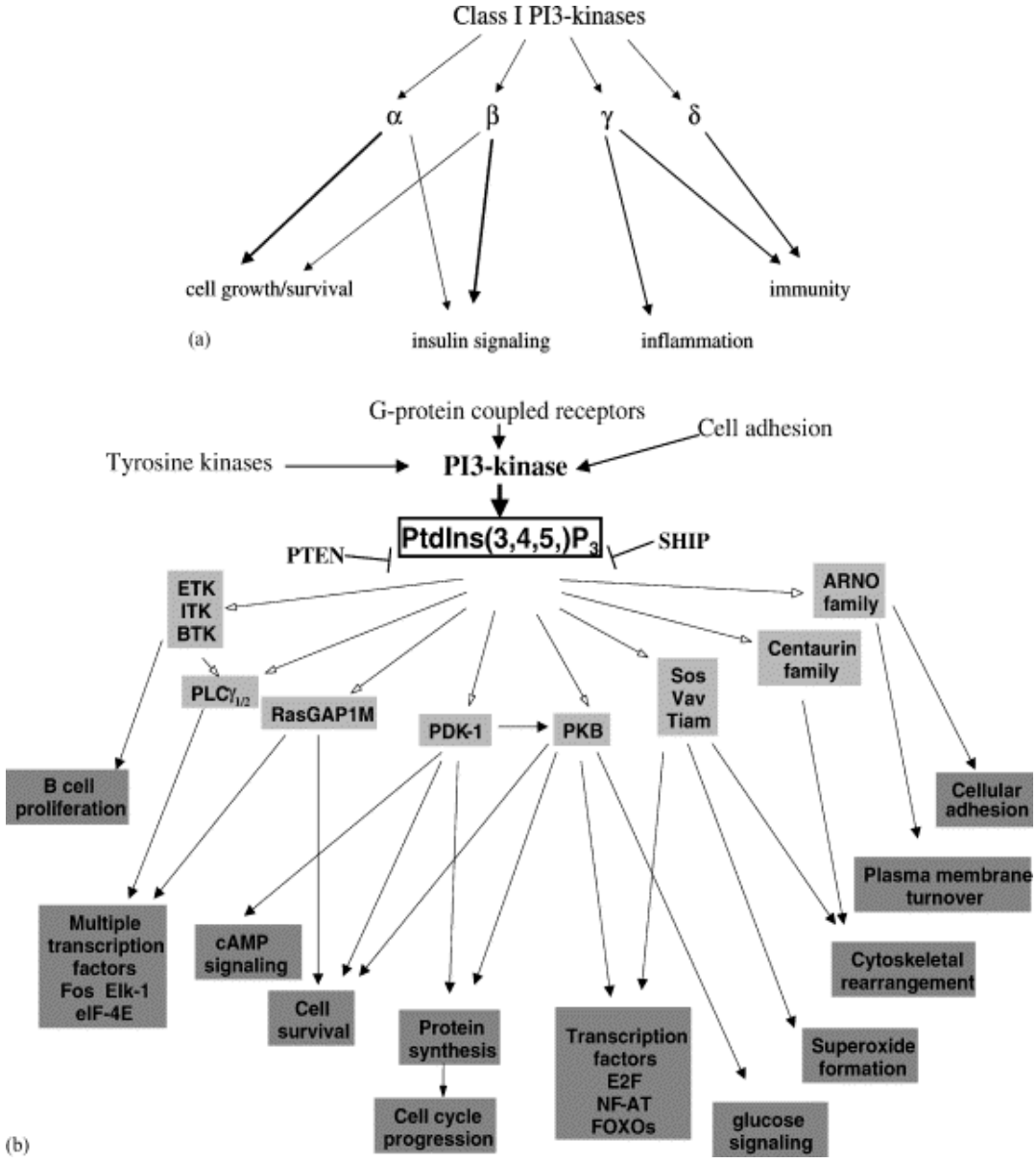


Figure 4: Downstream targets of the PI3 kinase pathway

modifying the localization of p27 by it's phosphorylation at threonine 157 on p27, preventing nuclear import and making p27 mostly cytoplasmic (52). Furthermore, when PKB/Akt phosphorylates p27 at threonine157, it impairs nuclear import of p27 and opposes p27 mediated G_1 arrest (53) (54). The sequence requirement for nuclear localization and growth inhibition of p27 was determined in Hela cells. The sequence is amino acids 153-168 and residues K168 and I169 were critical for nuclear localization (55). Another study by Sekinoto et al has also demonstrated that Akt phosphorylates p27 at threonine 157 and that this prevents nuclear import of the protein. This group located the nuclear localization signal of p27 to the c-terminal end of the protein specifically the classical bipartite-type basic region plus amino acids 152-168. This group attempts to show that the scaffold protein 14-3-3 competes with the importin protein α5 for binding to threonine 157 phosphorylated p27, and prevents it from entering the nucleus. In the absence of 14-3-3, p27 is imported via this protein (56).

Ras Proteins

Ras proteins have been shown to influence p27 localization through the Ras/Raf/Mek/Erk pathway (33). Also Ras is involved in the activation of PI3 Kinase through G protein coupled receptors (50). Ras proteins are members of a large Ras super family of structurally and functionally conserved GTPases. The carboxyl terminus of all Ras proteins has a highly conserved distinct sequence, a caax motif and a hypervariable domain that promotes association with the plasma membrane. Differences in the membrane localization of Ras might correlate with the specificity of different proteins in this family. Mammalian cells contain at least three Ras proto-oncogenes: H-Ras, K-Ras

and N-Ras. H-Ras and K-Ras were first discovered as viral (v-Ras) oncoproteins of the Harvey and Kirsten murine sarcoma viruses that were capable of cellular transformation. H-Ras is highly expressed in the skin and skeletal muscles, K-Ras in the colon and thymus, and N-Ras in male germinal tissue and thymus. The K-Ras gene is mutated in non-small lung cancer (33%), colorectal cancer (44%) and pancreas cancer (90%). N-Ras is mutated in melanoma (13%), liver cancer (30%), and myelogenous leukemia (30%). H-Ras is mutated in bladder cancer (10%), and kidney cancer (10%) (57).

Ras Effectors

There are three main Ras effectors in cells, RAF kinase, RAL-GEFs and PI3-K, which bind the same region of Ras-GTP the 32-40 amino acid domain. Ras-GTP can bind and activate the catalytic subunit of PI3 kinase, which can then activate downstream targets like Akt. Ras is often required to transmit signals from receptor tyrosine kinases (57). If Ras function is inhibited by an anti-Ras antibody or a dominant- negative mutant Ras, cell proliferation or differentiation responses normally induced by the activated RTK are inhibited. If constitutively active Ras is transfected into some cells, cell proliferation or differentiation is increased. Ras has been shown to activate separate signaling pathways including the Raf/Mek/Erk/ pathway and the PI3K pathway. It has been demonstrated that in cells continuously exposed to growth factors, that Ras is needed in two phases of the G_0 to S interval (58). This is evident by studies conducted with anti-Ras antibody or dominant negative Ras mutants that can be introduced to cells several hours after serum stimulation and still block S phase. Also Ras antibodies blocked the cell cycle when given before serum stimulation (59). Several researchers found that Erk is active

only during the initial part of prolonged Ras activity and that active Erk is not detectable during the mid to late phases of G_1. This might suggest that the Erk pathway is not responsible for this part of the cell cycle (60). This observation raises the possibility that Ras targets different effectors for the various phases of the cell cycle. PI3k was discovered to be active during G_0/G_1 transition and mid to late G_1. Inhibiting PI3k activity during G_0/G_1 had no effect on cell cycle progression but did affect S phase entry when it was inhibited in mid and late G_1 (61). Lastly, Ras has been observed to be active throughout the cell cycle but only stimulated cyclin D1 and promotes cell cycle progression during G_2 phase (58). It has previously been shown that activation of the Ras/Raf/Mek/Erk pathway in breast cancer cells causes p27 to be exported to the cytoplasm (33) and that constitutive activation of this pathway prevents arrest by antiestrogens. Mitogen stimulation of quiescent cells causes two peaks of Ras activation, one on entry into G_1 phase and associated with Raf pathway, and the second associated with PI3 kinase pathway at mid G_1 phase. It has been shown that activated Ras is essential for up regulation of cyclin D1 and down regulation of p27 (57). A summary of Ras targets is shown in figure 5.

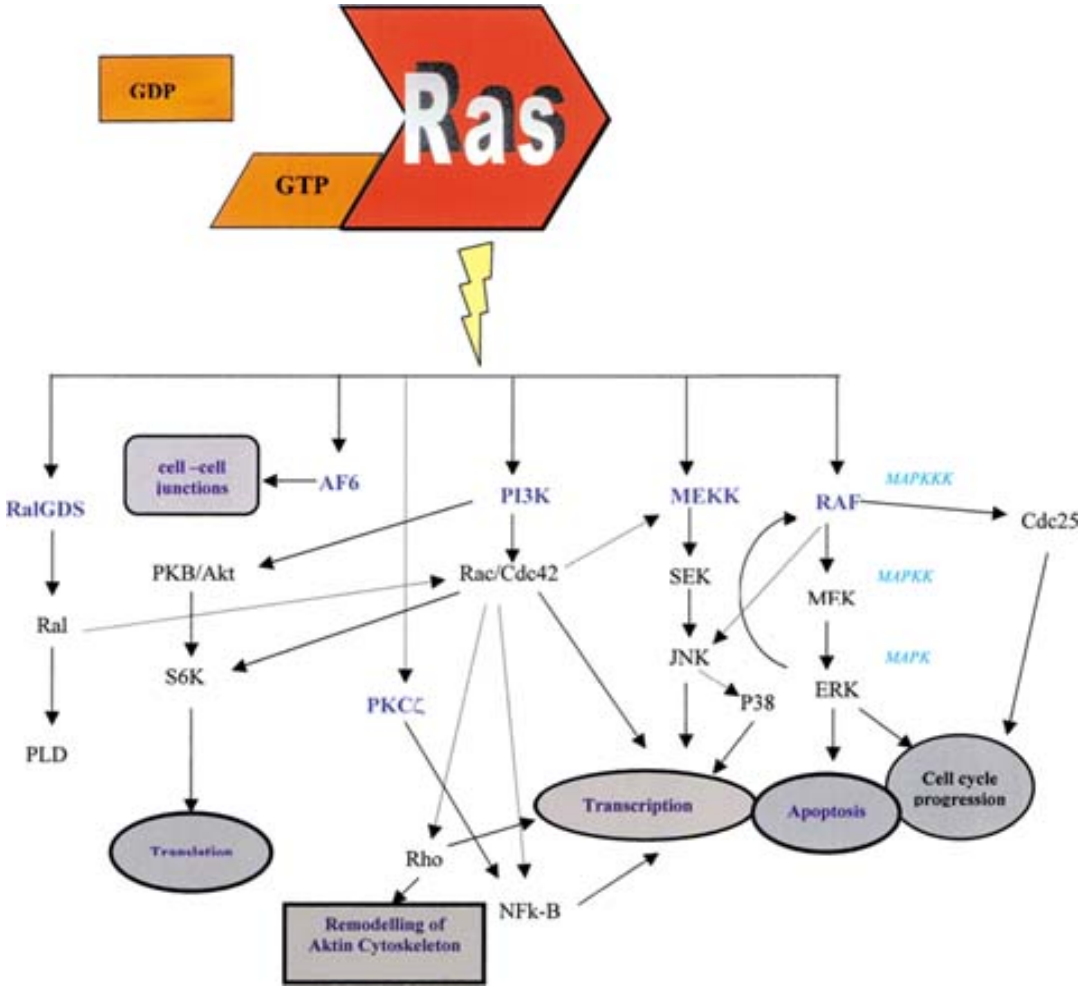


Figure 5. Ras downstream targets and actions

II. HYPOTHESIS AND RATIONALE

The study of the cell cycle inhibitor p27 and its function in cancer cells has become a complicated story. p27 knockout mice studies have provided insight into the necessity of this protein, but have also raised many questions, primarily, what is the main function of p27 in breast cancer and other cancer types. Not only is the stability of p27 important, but also studies have recently emerged showing that the cellular location of p27 may be involved in cancer development. The hypothesis that p27 has divergent functions based on its location in the cell may explain large differences in the phenotypes of p27-/- and p27+/- mice. Although recent studies have sought to discover the signal pathways that influence p27 localization, there remains much to be clarified.

The mammary tissue of p27 heterozygote mice is hyperproliferative and susceptible to tumors in some studies. Translational mechanisms rather than transcriptional processes more often regulate p27 levels in the mammary gland, and many studies have suggested that signal transduction pathways like PI3 kinase/Akt and Ras/Raf/Mek/Erk have an effect on both p27 localization and degradation. The hypotheses to be tested in this study are 1) Active Akt effects the localization of p27 in MCF-7 breast cancer cells 2) active Akt effects the stability of p27 in these cells 3) active Akt effects the stability of p27 differently in the nucleus versus the cytoplasm 4) estrogen effects p27 stability and localization through the PI3 kinase/Akt pathway.

III. MATERIALS AND METHODS

Reagents and Antibodies

Cell culture media and antibiotics, 17β estradiol (E2), fetal bovine serum (FBS), anti-flag M2-agarose affinity gel, monoclonal (M2) and rabbit polyclonal anti-flag antibodies were from Sigma Chemical Company (St. Louis, MO). ICI 182,780 is supplied by Dr. Alan Wakeling at Zeneca Pharmaceuticals (Alderly Park, Chashire, UK). Protein A/G beads were from Santa Cruz (California). Monoclonal anti-actin was from Roche Applied Science (Indianapolis, IN). Tubulin and fluorochrome-labeled goat anti mouse secondary antibody 488 were from Molecular Probes (Eugene, Oregon). E2F-1 antibody was from Neomarkers/Labvision (Freemont, Ca). Tran $[^{35}S]$ label was from ICN (Irvine, CA).

Cell Culture and Transfections

MCF-7 cells were a gift from R.P Shiu and maintained in Dulbecco's Modified Eagles Medium with 2.5% FBS and penacilin/streptamicin. Cells were growth arrested by 2 days of culture in phenol red free Dulbecco's Modified Eagles Medium with .1% FBS and 20 nM ICI 182, 780. Chemical stock solutions were prepared in ethanol (ICI 182,780, and 17β estradiol), and control cultures received equal amounts of solvents as vehicle controls where appropriate. Plasmid vectors for MryAkt were from (Antonio Cuadrado). M. Cobb provided vectors for constitutively active ERK kinase. Plasmids for flag-p27 (wild-type and T157A mutant) were from Naoya Fuijita. C..M. Counter provided the Ras V12S35 plasmid. Transfections were carried out using LipofectAMINE

PLUS transfection reagent from Invitrogen (Carlsbad, CA), using .2 μg of flag-p27 and .4 μg of other vectors or .6 μg of total DNA to cells seeded in 6 well plates.

Cell Lysis, Western Blotting and Immunoprecipitations

Cells were collected with PBS/EDTA in microcentrifuge tubes and centrifuged for 5 minutes at 2.5 RPM. Cell pellets were lysed in ice-cold .5% Nonident P-40, and triton X-100 buffer with 1 mM EDTA, 1mM NaOV4, 10 mM NaF, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, with centrifugation at 11, 000 G for 10 minutes to remove cellular debris. Protein samples were equalized using the Bradford Assay from BIORAD (Hercules, CA) and read with the spectrophotometer machine. Western blotting samples were prepared with an equal amount of protein (50-100μg) and 25% of the sample consisted of Lammeli sample buffer. Equal volumes (20-25 μl) of protein samples were loaded onto a 12% polyacrylamide gel. The gel was run for 1.5 hours then transferred to nitrocellulose, washed with TBST then blocked in .5%casein/TBST for thirty minutes. The blot was incubated with flag-p27 rabbit polyclonal antibody diluted 1:1000 in blocking buffer overnight. After primary antibody, the blot was washed three times with TBST then incubated with anti-rabbit secondary antibody in blocking buffer for one hour. Then blot was washed three times for five minutes with TBST and ECL chemilumenecent reagent from Amersham (Buckinghamshire, England) was added to the blot for five minutes before blot was exposed to Kodak or Fuji film. Analysis of protein bands on the films was determined with Kodak 3.5 imaging software, and graphed using Prism Graph pad software 4.0. Immunoprecipitations were performed with 2 μl/sample

of Flag M2 affinity agarose (50% slurry in PBS) with 15 µl A/G beads per sample in addition to 150 µl of lysis buffer plus equal amounts of protein lysate.

Cellular Localization of p27 by Fractionation

Nuclear/cytoplasmic cell fractionation was carried out by lysis in hypotonic buffer with .02% digitonin. Samples were then vortexed lightly once per minute for five minutes, then centrifuged at 1000G for five minutes to separate the nuclear fraction from the cytoplasmic fraction. After centrifugation, the cytoplasmic fraction was removed and placed into new microcentrifuge tubes then centrifuged at 10,000G for ten minutes to remove cellular debris. Nuclear fractions were lysed with hypotonic buffer then centrifuged for five minutes at 1000 G, then the supernatant was discarded and the remaining nuclear fraction was freeze thawed then lysed in 500 mM NaCl and ice-cold NP-40 lysis buffer as described previously.

Cellular Localization of p27 by Immunoflorescence

MCF-7 cells grown on glass cover slips were transfected with the flag-p27 plasmid with MryAkt, pcDNA3, dnAkt, or RasV12S35 plasmids. After forty hours, cells were washed with PBS then fixed with 3% paraformaldehyde for twenty minutes. After fixation, cells were washed with PBS then permeablized with .5% Triton in 1% BSA for five minutes. After permeablization, cells were washed three times with PBS then blocked with blocking buffer (1% goat serum +1%BSA) for thirty minutes. Then slides were incubated with primary antibody (anti-flag M2 monoclonal .2 µg/ml) overnight in 4^{o}C. After primary antibody cells were washed in PBS three times for five minutes then incubated for two hours with secondary fluorochrome-labeled goat anti- mouse antibody.

The cover slips were then mounted on slides using DAKO mounting medium, and nuclear versus nuclear/cytoplasmic flag-p27 was counted visually on an Olympus IMT-2 fluorescent microscope. For quantification, a minimum of three hundred cells were counted for each determination and results were derived from at least three independent experiments.

Pulse and Pulse Chase Analysis of p27

MCF-7 cells in 6-well plates were transfected with flag-p27 or flag-T157Ap27 (mutant p27) with either of the following, MryAkt, pcDNA3, or dnAkt. 40 hours post transfection, methione/cysteine minus medium was replaced as the cell culture medium for 30 minutes. The cells were then labeled with 50 μCi/well of [^{35}S] methionine added to medium (without methionine or cysteine) for one hour. For the chase, monolayers were washed once with fresh complete medium and incubated for the indicated times then collected for fractionation or lysis. For the pulse, 50 μCi/well of ^{35}S methionine was added to cells on 6 well plates and at the indicated times, cells were washed with fresh complete medium then collected for fractionation or lysis. Flag-p27 was immunoprecipitated from nuclear/cytoplasmic lysates or whole cell lysates as explained above. An equal amount of ^{35} S methionine labeled lysates was calculated from quantitative values obtained from TCA precipitation of 2 μl of each sample analyzed by a scintillation counter

p27 Stability Determined by Cyclohexamide Experiments

MCF-7 cells in 6 well plates were cotransfected with flag-p27 and other indicated plasmids. 40 hours post transfection, cells were treated with 10 μg/ml cyclohexamide for

the indicated time points then cells were collected and lysed as explained previously and analyzed by western blotting.

Statistical Analyases

Performed with Graph Pad Prism Software 4.0

IV. RESULTS

MryAkt Keeps p27 Mostly Cytoplasmic

We examined the role of activated Akt in the nuclear/cytoplasmic localization of p27. In order to activate the Akt pathway exclusively, MCF-7 breast cancer cells were transfected with MryAkt plasmid and also flag-p27 plasmid. p27 localization was enumerated by immunostaining with anti-flag antibodies. MryAkt expression in the cells causes flag-p27 to be significantly ($p<.003$) more cytoplasmic (figure 6) compared to control cells expressing flag-p27 and control plasmid pcDNA3 (compare bars one and two from left). The amount of cytoplasmic p27 is also significantly decreased ($p<.004$ compare bars three and four from right to second bar from right) in cells expressing flag-p27 and the dominant negative inactive Akt (dnAkt) plasmid in growing medium with serum, or with estrogen. Estrogen is a potent mitogen for MCF-7 cells, and a proven activator of the PI3 Kinase/Akt pathway, and the Ras/Raf/Mek/Erk pathway (39), (40), (41). Cells expressing dnAkt then treated with estrogen showed less cytoplasmic p27, compared to cells expressing flag-p27 and MryAkt. The dnAkt results suggest that growth factors like serum or estrogen cannot effect p27 localization when the Akt pathway is inhibited. Furthermore, previous studies (52), (53), (54) have demonstrated that, the cytoplasmic retention of p27 is theoretically due to phosphorylation of p27 at threonine 157 by activated Akt, which prevents nuclear translocation of p27. In order to determine if this phosphorylation site is required for the cytoplasmic retention of p27, cells were transfected with MryAkt and with flag-T157Ap27 plasmid, in which the Akt phosphorylation site on p27 is mutated and cannot be

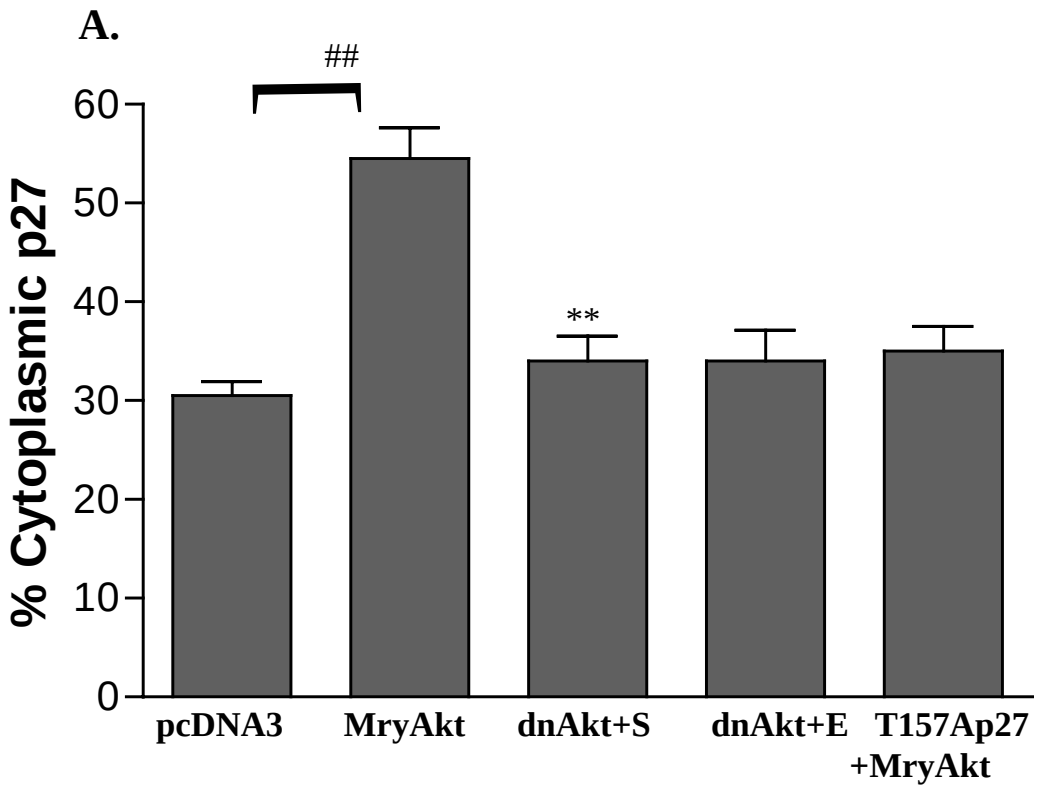


Figure 6: MryAkt increases cytoplasmic p27.
(A) MCF-7 cells were transfected with flag-p27 and one of the following cotransfectants: pcDNA3, MryAkt, dnAkt (in complete medium with serum (S)), dnAkt with E2, or T157Ap27 with MryAkt. After 40 hours of growth arrest in medium with .2% FBS and 20 nM ICI, the localization of flag-p27 was analyzed by immunostaining with anti-Flag antibodies. The proportion of cells exhibiting cytoplasmic p27 was enumerated. The values for the experiment are devrived from three independent experiments and are givein as the mean + S.D. Statistical analysis was based on a two-tailed t test. # #, significantly different from controls at $p<.003$, ** $p<.004$ for dnAkt+S, dnAkt+E, and T157Ap27 and MryAkt.

phosphorylated by active Akt. In figure 6 (far right bar on graph), when cells express the T157Ap27, the amount of nuclear p27 is significantly ($p < .004$) reduced to control levels. T157Ap27 is significantly less cytoplasmic than wild type flag-p27 in cells coexpressing MryAkt (compare far right bar with second bar from left).

Fractionation of MCF-7 cells transfected with MryAkt plasmid and flag-p27 plasmid verified that active Akt does increase cytoplasmic p27 and decrease the amount of nuclear p27 protein (figure 7, compare lanes one and three, two and four) compared to cells transfected with flag-p27 and pcDNA3. Cells expressing flag-p27 plasmid and dnAkt plasmid show an equal distribution of p27 in both compartments (lanes 5 and 6). In agreement with the immunoflorescent experiment data, cells expressing MryAkt and flag-T157Ap27 show less cytoplasmic flag-p27 than wild type p27 transfected cells with MryAkt (compare lanes 3&4 of panel A with lanes 3&4 panel B).

The Ras/Raf /Mek/Erk pathway has previously been shown to cause rapid export of p27 from the nucleus when this pathway is activated. The Ras/Raf/ Mek/ Erk pathway causes export of p27 by a Crm-1 dependent pathway when it is phosphorylated on Serine 10 (33). In figure 7B, constitutively active Ras and Mek plasmids transfected into cells with flag-p27 showed increased cytoplasmic p27 as expected.

MryAkt Prevents Newly Synthesized p27 from Entering the Nucleus.

In figures 6&7, p27 increases in the cytoplasm when Akt is activated. We wanted to further investigate whether Akt affected the localization of nascent p27 as well. MCF-7 cells were transfected with flag-p27 plasmid with MryAkt plasmid, or flag-p27 with control plasmid. After 40 hours, the transfected cells were pulsed with ^{35}S methionine for

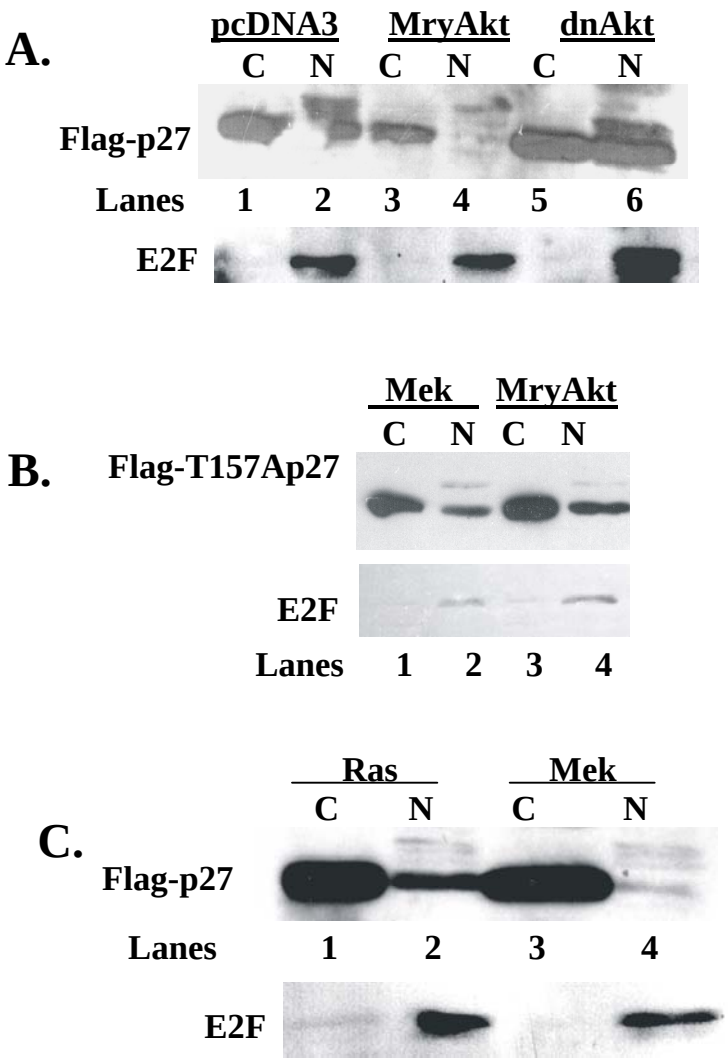


Figure 7: MryAkt, Ras, and Mek increase cytoplasmic p27. (A) Active Akt increased cytoplasmic p27 compared to controls. MCF-7 cells were transfected with flag-p27 and one of the following cotransfectants: (A)pcDNA3, MryAkt, dnAkt, or (B) T157Ap27 with MryAkt (C) Constitutively active Ras and Mek. After 40 hours of growth arrest in medium with .2% FBS and 20 nM ICI, the localization of flag-p27 was analyzed by western blotting and staining with anti-flag antibody. E2F protein is a nuclear compartment control. (B) T157Ap27 is more nuclear compared to wildtype (compare lanes 4 with lane 4 of panel A). (C) Ras and Mek increase cytoplamic p27, Compare lanes 1and 3 with panel A lane 1.

two, four and six hours. At the completion of each time, cells were collected and fractionated. The quantitative results based on densitometry from three experiments are presented as graphs in figures 8A and B, which display the percentage of p27 in each cellular compartment at the indicated times. Figure 8C shows a representative autoradiograph. Figure 8A indicates that MryAkt decreased the amount of nascent nuclear flag-p27 by six hours. In contrast, flag-p27 in control plasmid expressing cells shows p27 increasing in the nucleus. The cytoplasmic compartment reflects the opposite; MryAkt causes cytoplasmic flag-p27 to increase (figure 8B), while control cells show a decrease in cytoplasmic p27. This data agrees with that obtained in figures 6 and 7.

The data obtained in figure 8 shows that flag-p27 increases in the cytoplasm in cells were Akt is activated. Whether Akt is affecting nuclear export or import of nascent flag-p27 is still in question. In order to clarify this issue, cells were transfected with flag-T157Ap27 (the phosphorylation mutant of p27) and MryAkt, or dnAkt or pcDNA3. If MryAkt is affecting import of flag-p27, then this mutant should behave opposite to wildtype flag-p27 i.e. not be retained in the cytoplasm. Figure 9A demonstrates that flag-T157Ap27 increases in the nucleus in contrast to wildtype flag-p27 (figure 8A) in Akt activated cells. In addition, flag-T157Ap27 decreases in the cytoplasm in figure 9B when coexpressed with MryAkt. DnAkt and pcDNA3 transfected cells coexpressing flag-T157Ap27 show this protein decreasing in the nuclear compartment in figure 9A, and slightly increasing in the cytoplasm in figure 9B. The cause of this phenomenon remains unclear and requires further investigation.

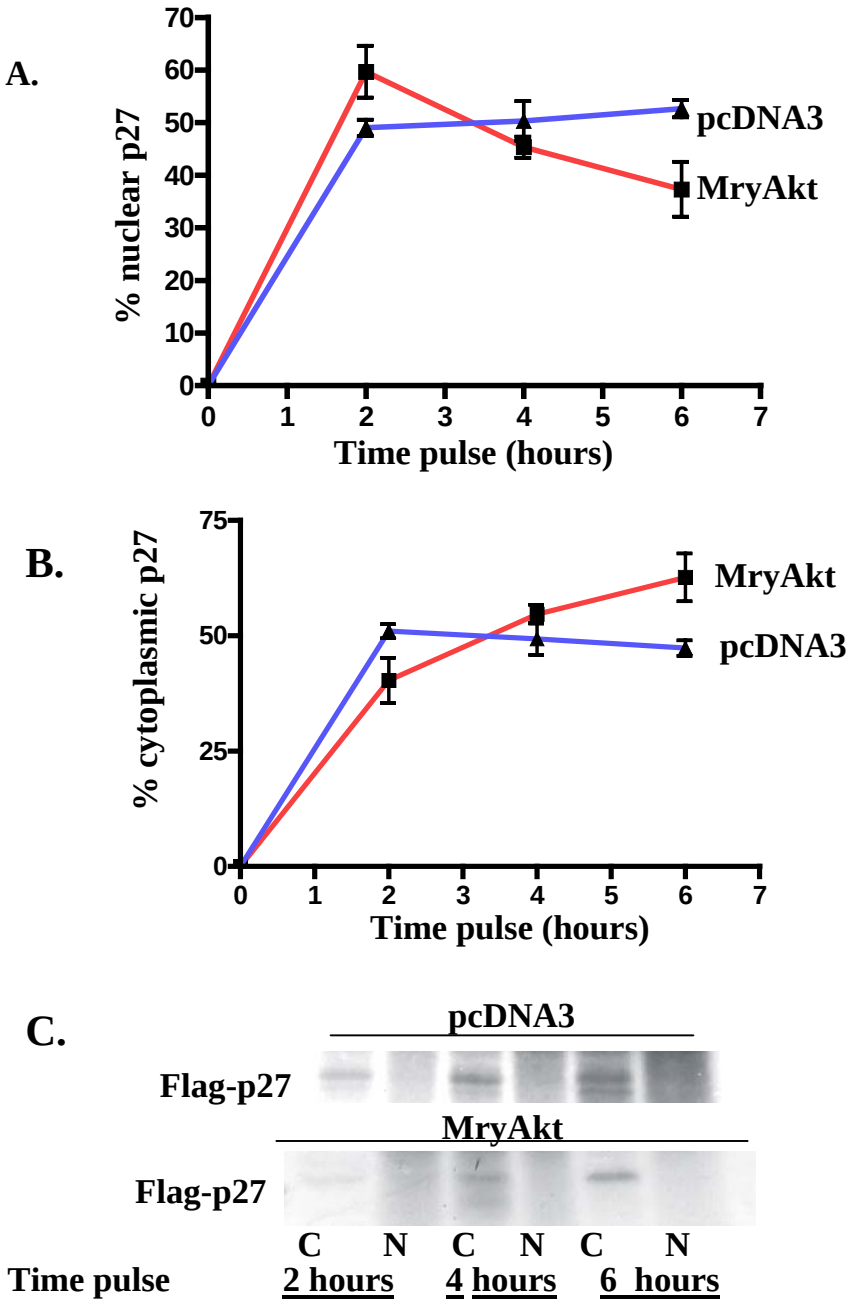


Figure 8: MryAkt increases nascent cytoplasmic p27
(C) Cells were transfected with flag-p27 plus MryAkt (bottom) or pcDNA3 (top) plasmids. After 40 hours the localization of new p27 was determined by pulse analysis with ^{35}S methionine. At the indicated times cells were collected and fractionated. The nuclear and cytoplasmic lysates were immunoprecitpitated with anti-flag antibody and resolved by SDS-PAGE. (A,B)The quantitative results based on densitometry are presented as graphs (C) Representative blot of one experiment: C=cytoplasmic fraction, N=nuclear fraction

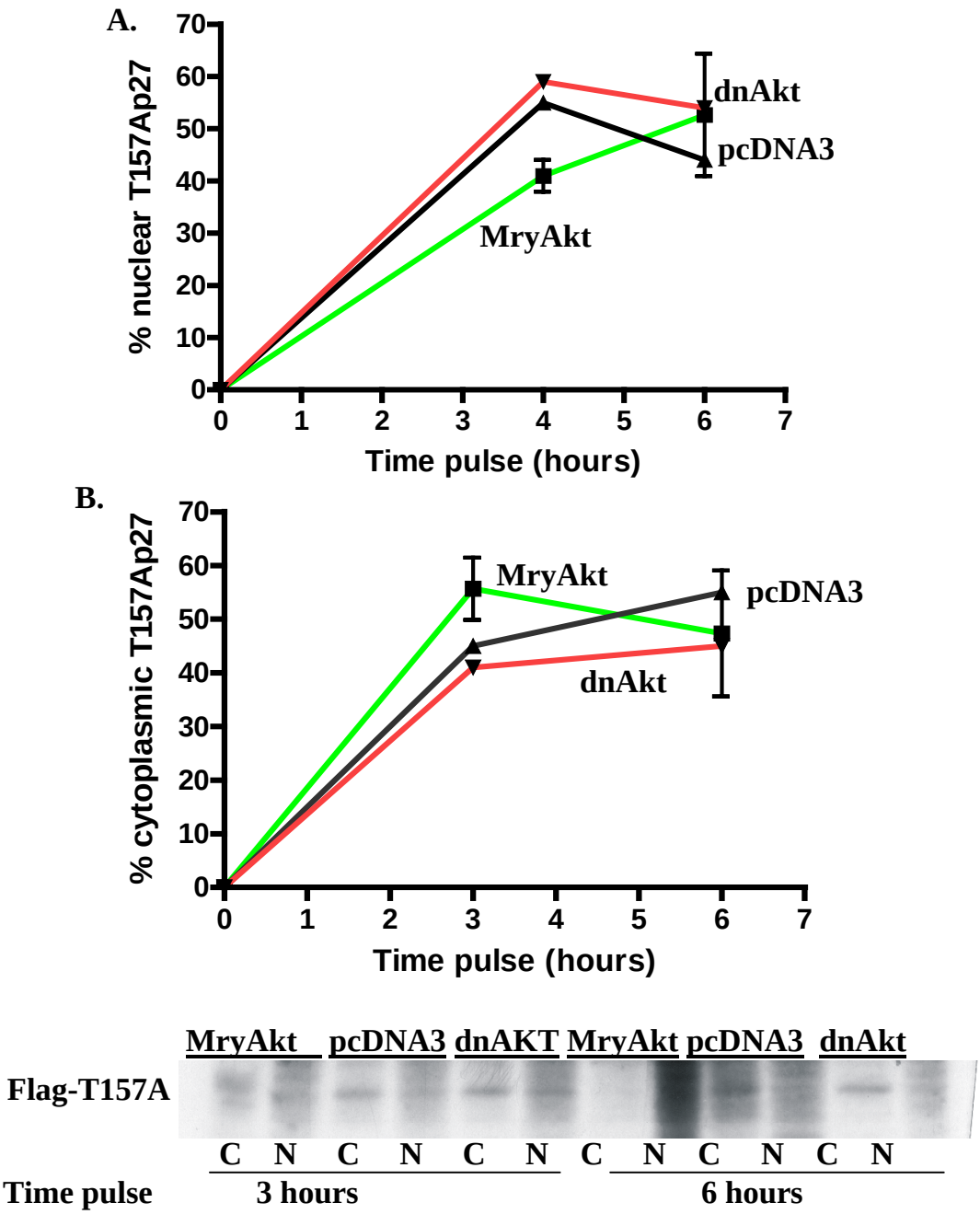


Figure 9: MryAkt increases T157A in the nucleus

(C) Cells were transfected with flag-T157Ap27 plasmid and pcDNA3 or dnAkt plasmid. Cells were pulsed with ^{35}S methionine and at 3 and 6 hours, cells were collected and fractionated. Flag-T157Ap27 was immunoprecipitated and from cytoplasmic and nuclear lysates. Immunoprecipitates were resolved by SDS-PAGE. (A,B) The quantitative results based on densitometry are presented as a graph (A) % nuclear T157Ap27 (B) % cytoplasmic (C) Representative blot: C=cytoplasmic fraction, N=nuclear fraction

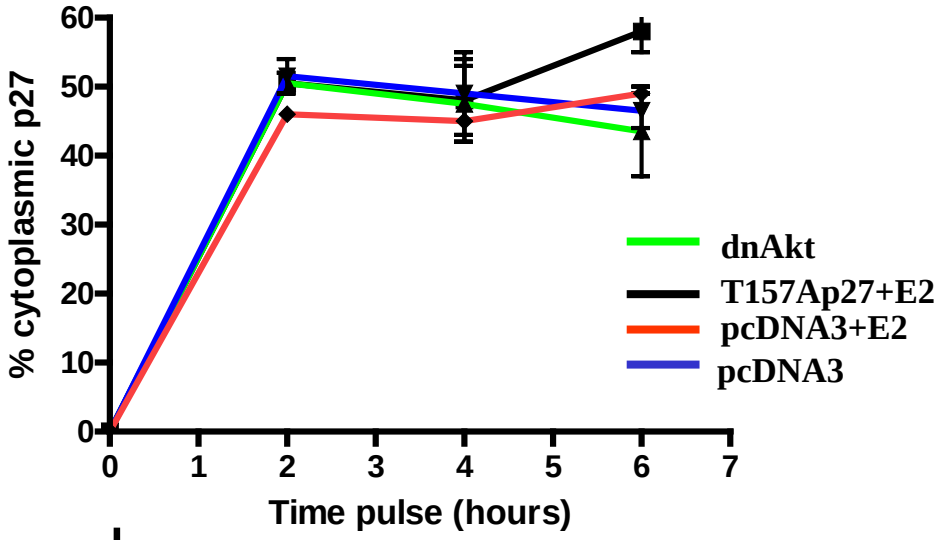
Estrogen Increases Nascent Cytoplasmic p27 by Activating Akt

Estrogen activates the PI3 Kinase/Akt pathway in MCF-7 cells (41), and is a potent mitogen; estrogen decreases p27 levels, and increases cyclin D protein (3). In this study we wanted to investigate the role estrogen plays in the localization of p27. Cells were transfected with flag-p27, or flagT157Ap27 and pcDNA3. Also some cells were transfected with dnAkt and flag-p27. Transfected cells were labeled with ^{35}S methionine as described above and fractionated. Estrogen was added to the cells at time of pulse where indicated in figure 10A. Estrogen caused a slight increase of cytoplasmic flag-p27 by 6 hours, compared to control cells with flag-p27 and pcDNA3 without estrogen treatment. DnAkt with estrogen showed decreased cytoplasmic flag-p27 (figure 10A green line). This suggests that overexpressing inactive Akt (dnAkt) can inhibit the ability of estrogen to influence p27 localization, and that estrogen must regulate p27 through this pathway.

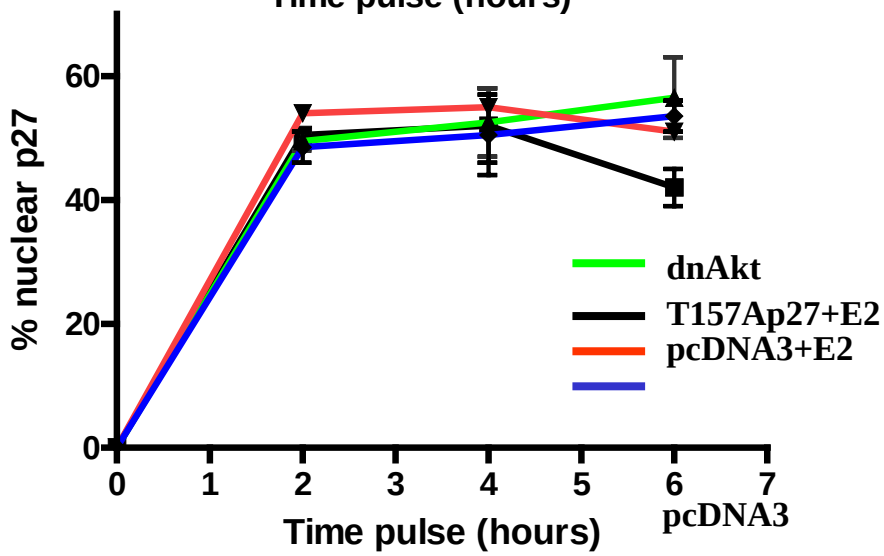
Surprisingly, cells transfected with flag-T157Ap27 and estrogens behave similar to wildtype flag-p27 with estrogen (figure 10A&B). T17Ap27 accumulates in the cytoplasm similar to wildtype (figure 10A compare black to red line on graph). Also T157Ap27 decreased in the nucleus (figure 10B compare black and red line). Previously it has been shown that estrogen can cause export of serine 10 phosphorylated p27 from the nucleus when mitogen is added via the Erk pathway. If T157Ap27 is entering the nucleus, then adding estrogen can increase its export.In estrogen treated cells, wildtype flag-p27 was prevented from entering the nucleus by activating Akt, which phosphorylates p27 on threonine 157.

Figure 10: Estrogen increases nascent cytoplasmic p27 through Akt
(C,D) Cells were transfected with flag-p27 and pcDNA3, or dnAkt, or T157Ap27 and pcDNA3. After 40 hours of growth arrest estrogen (E2) was added to the indicated samples simultaneously with ^{35}S methionine. At the indicated times cells were collected and fractionated. Fractionated lysates were immunoprecipitated with anti-flag antibody. Immunoprecipitates were resolved by SDS-PAGE. (A) The quantitative results based on densitometry are presented as a graph % cytoplasmic flag-p27 or flag-T157A, (B) % nuclear flag-p27 or flag-T157Ap27 (C,D) Representative blot of one experiment. C=cytoplasmic fraction, N=nuclear fraction

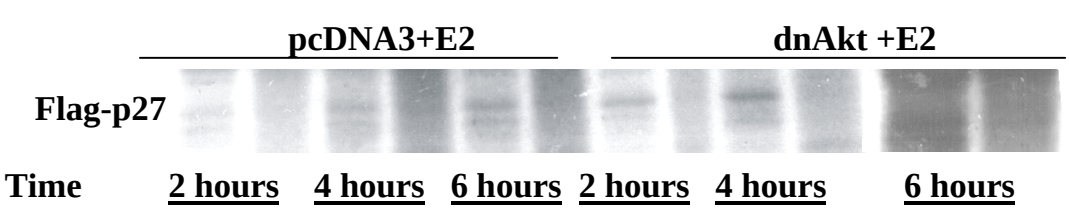
A.


% cytoplasmic p27
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
Time pulse (hours)
dnAkt
T157Ap27+E2
pcDNA3+E2
pcDNA3

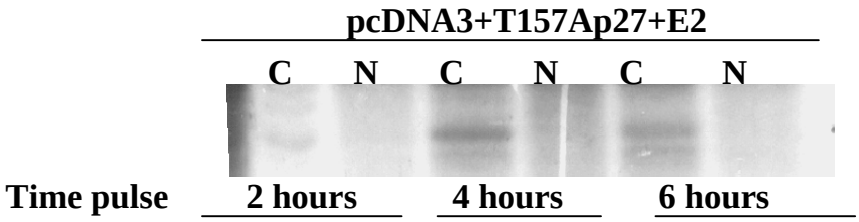
B.


% nuclear p27
60
40
20
0
0 1 2 3 4 5 6 7
Time pulse (hours)
pcDNA3
dnAkt
T157Ap27+E2
pcDNA3+E2

C.


pcDNA3+E2
dnAkt +E2
Flag-p27
Time
2 hours
4 hours
6 hours
2 hours
4 hours
6 hours

D.


pcDNA3+T157Ap27+E2
C N C N C N
Time pulse
2 hours
4 hours
6 hours

MryAkt Stabilizes Flag-p27

The stability or degradation rate of p27 is important for proper MCF-7 cell cycle function. Interfering with p27 stability can alter the cell cycle in these cells and other cell types. Since p27 levels are often affected by signal transduction pathways in the cell, we also wanted to investigate whether activating the Akt pathway could affected the stability of flag-p27 in MCF-7 cells.

Analysis of p27 degradation by activated Akt was conducted by transfecting cells with flag-p27 and MryAkt plasmids. After growth arrest, the cells were treated with cyclohexamide (10 μg/ml) for the indicated times for two, five and eight hours. The cells were collected and western blotted with anti-flag antibody. This experiment was repeated three times with comparative results. Figure 11B displays the western blot from cells expressing MryAkt and flag-p27 (left panel), or pcDNA3 and flag-p27 (right panel). The quantitative results based on densitometry are represented as a graph in figure 11A. Figure 11A&B show that MryAkt expressing cells have stabilized flag-p27 protein, compared to cells expressing flag-p27 and pcDNA3 which show a steady decrease in flag-p27 levels over time. MryAkt expression stabilizes p27 protein but has no effect on actin levels.

In order to determine if the Akt phosphorylation mutant of p27, T157Ap27, has any role in stabilization of p27, cells were transfected with flag-T157Ap27 plasmid with MryAkt plasmid. As shown in figure 11C, and in the graph in figure 11A, cells expressing flag-T157Ap27 were equally stable compared to wild type. This result

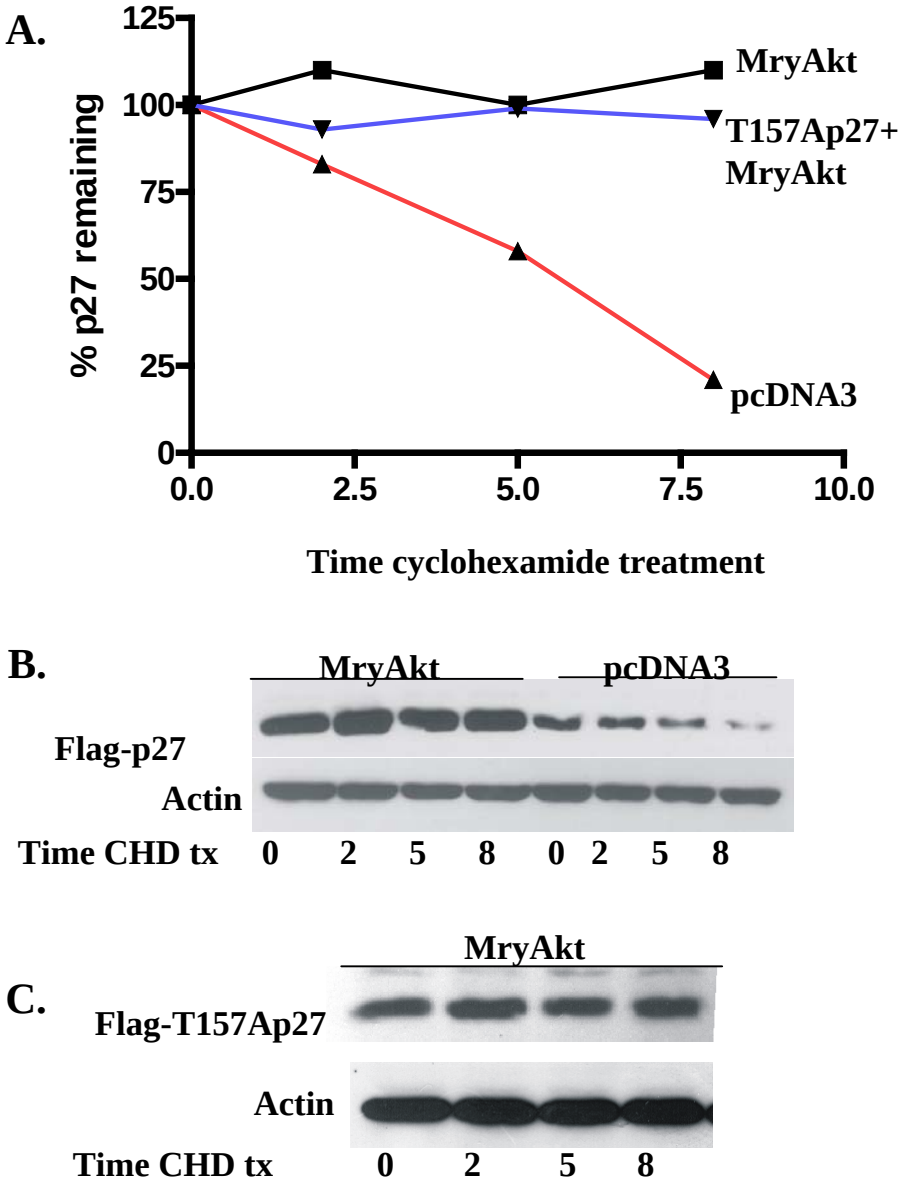


Figure 11: p27 degradation following cyclohexamide treatment
(B) Cells were transfected with MryAkt and flag-p27 plasmids or pcDNA3 and flag-p27, or (C) T157Ap27 and MryAkt. (B&C) After 40 hours, cells were treated with 10 ug/ul cyclohexamide for the indicated times prior to lysis. Protein lysates were analyzed by western blotting with anti-flag antibody. CHD=cyclohexamide, tx=treatment. The experiment was repeated three times with comparative results (A) The quantitative results based on densitometry are presented as a graph

suggests that this Akt phosphorylation site has no role in the stability of p27, and is only important in the cellular localization of p27.

The degradation results of flag-p27 following cyclohexamide treatment were verified by ^{35}S methionine labeling pulse chase experiments. MCF-7 cells were transfected with the flag-p27 with MryAkt plasmid, or pcDNA3 plasmid. Transfected cells were labeled with ^{35}S methionine for one hour then chased with complete medium (without ^{35}S methionine) and collected at the indicated times. ^{35}S methionine labeled protein lysates were used to immunoprecipitate flag-p27. Immunoprecipitates were resolved by SDS-PAGE and figure 12B shows a representative autoradiograph. The experiment was repeated three times and the graph in figure 12A displays the quantitative results of those experiments. The pulse chase labeling results are in agreement with the results obtained from the cyclohexamide experiments. Figure 12A shows that cells expressing MryAkt were able to stabilize flag-p27 protein up to eight hours, while cells expressing control plasmid show flag-p27 degrading over time.

Since the mutant of p27, T157A, was stable in the presence of MryAkt in cyclohexamide experiments, we wanted to investigate this concept by pulse chase analysis also. In figure 13, cells were transfected with T157Ap27 plasmid with MryAkt plasmid, then subjected to pulse chase analysis and resolved as explained above. The autoradiograph (figure 13B) of flag-T157Ap27 and the graph in figure 13A shows this protein accumulating in the cells, before it degrades by eight hours. The Akt phosphorylation site on p27, theoinine 157, does not seem to have a role in the degradation of p27, and this data suggests that this mutation might interfere with

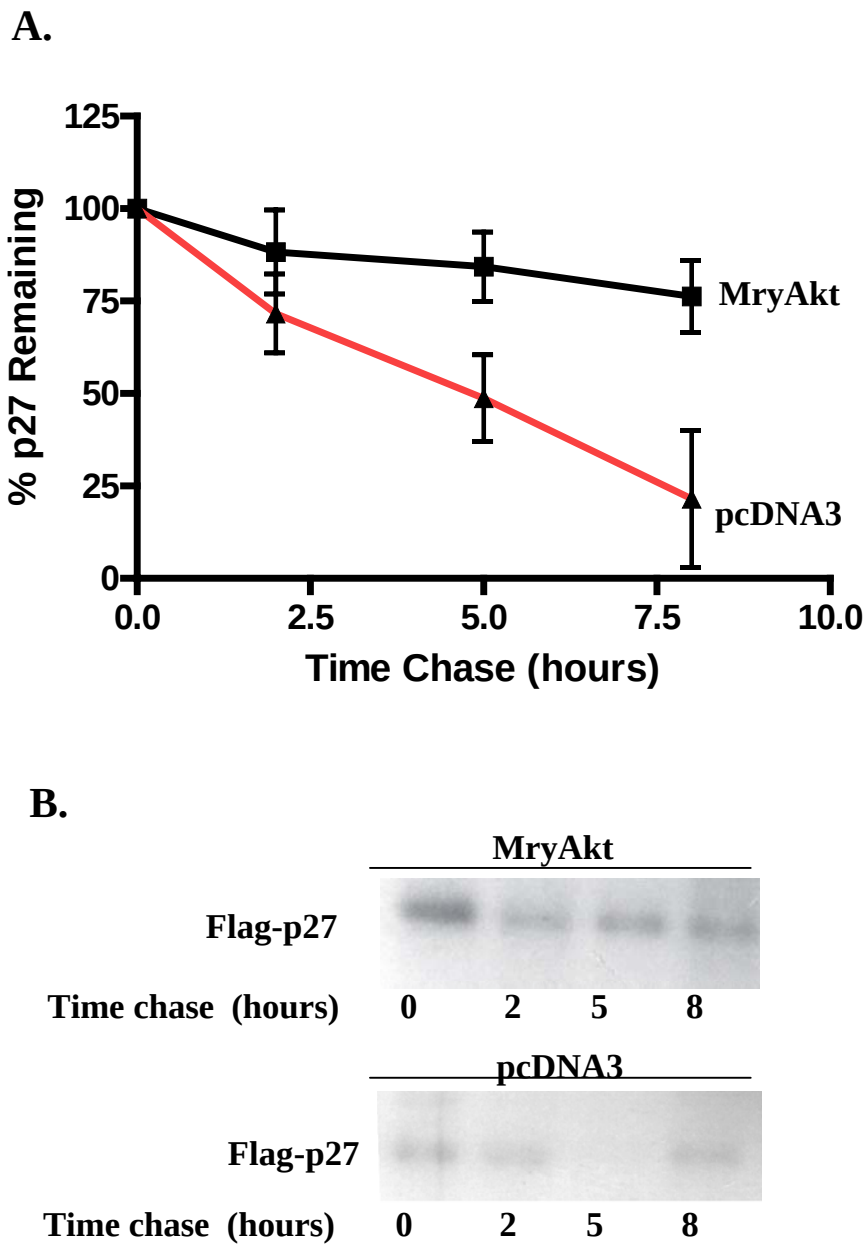


Figure 12: MryAkt stabilzes flag-p27 in vivo
(B) Cells were transfected with flag-p27 and MryAkt or pcDNA3 plasmids. After 40 hours, cells were labeled with ^{35}S methionine for 1.5 hours then chased with complete medium and collected at the indicated times. Cell lysates were immunoprecipitated with anti-flag antibody and resolved by SDS-PAGE (A) The quantitative results of three experiments based on densitometry are presented as a graph. (B) Representative autoradiograph of one experiment

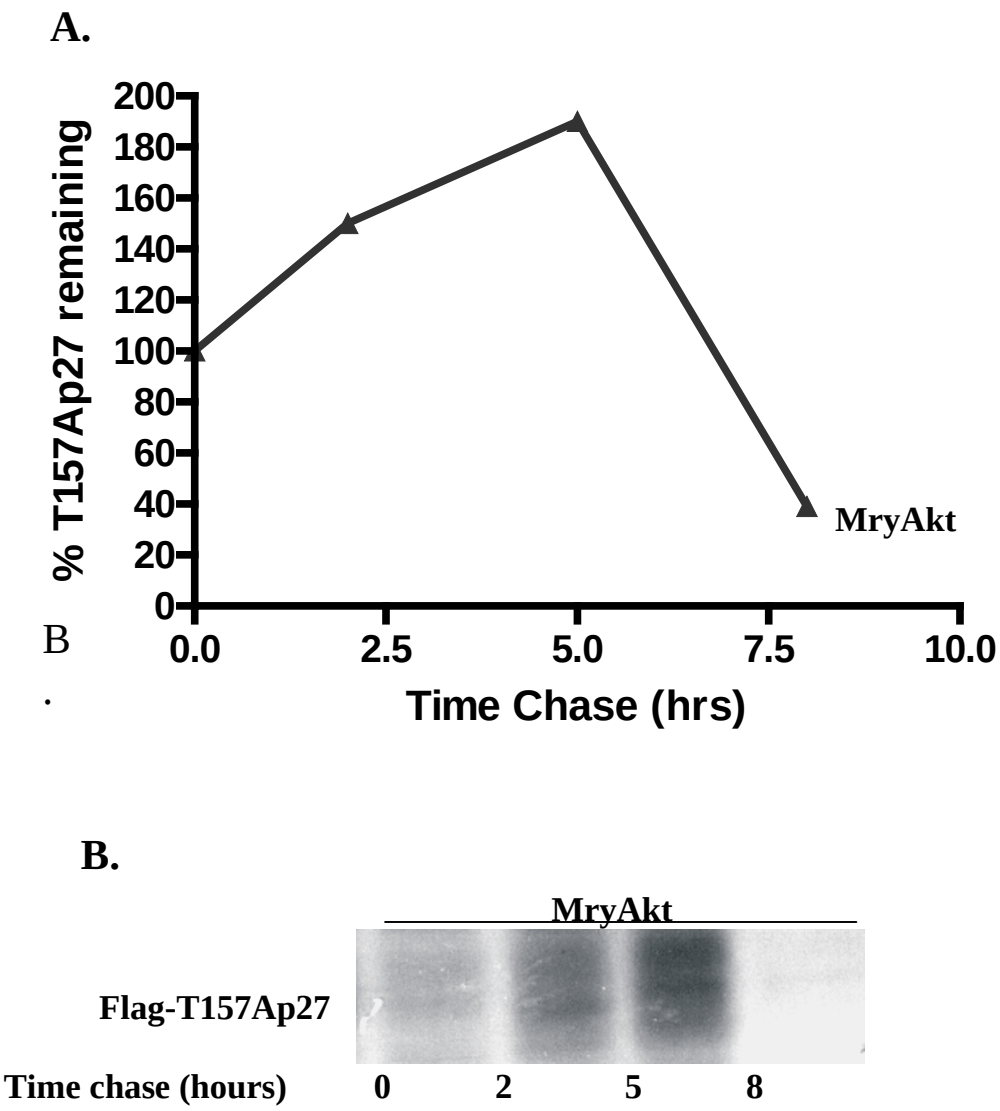


Figure 13: MryAkt stabilizes T157A p27
(B) Cells were transfected with the indicated flag-T157Ap27 plasmid MryAkt. After 40 hours cells were pulsed with ^{35}S methionine for one hour then chased for the indicated times. Lysates were immunoprecipitated with flag antibody then resolved by SDS-PAGE.
(A) Quantitative results based on densitometry are presented as a graph.

degradative mechanisms in the cell, shown by the accumulation and subsequent decrease of this protein in the cell.

MryAkt Has no Effect on Cytoplasmic p27 Degradation.

Since activated Akt stabilizes p27 protein, we sought to investigate which cellular compartment; the nucleus or cytoplasm, contained more stable p27. Cells were transfected with flag-p27 with MryAkt or pcDNA3, then labeled with ^{35}S methionine as explained previously. Cells were collected at zero, three and six hours and fractionated to separate the cytoplasmic compartment from the nucleus. The experiment was repeated two or three times and the collective quantitative densitometry results are shown by the graph in figure 14A. Figure 14B displays a representative autoradiograph of one experiment. In figure 14, flag-p27 was immunoprecipitated from the cytoplasmic lysate. MryAkt expressing cells show flag-p27 degrading at a similar rate in the cytoplasm, as in the whole cell lysate (compare graph in figure 14A with graphs in figures 11A and 12A). In contrast, flag-p27 in control plasmid expressing cells degrades slower in the cytoplasm than in the whole cell lysate (compare pcDNA data in figures 14A to figures 11A and 12A). This discrepancy in degradation rate for flag-p27 in pcDNA expressing cells in the cytoplasm compared to the whole cell lysate, could be due to the different distribution of p27 in the cell. When Akt is activated, there is more p27 in the cytoplasm than in control cells. This concept is further explained in the discussion. The T157Ap27 mutant accumulates in the cytoplasm and the whole cell lysate (compare figures 14A and 13A). This T157A mutation may alter degradative mechanisms in the cell, resulting slower degradation and subsequent accumulation of this protein.

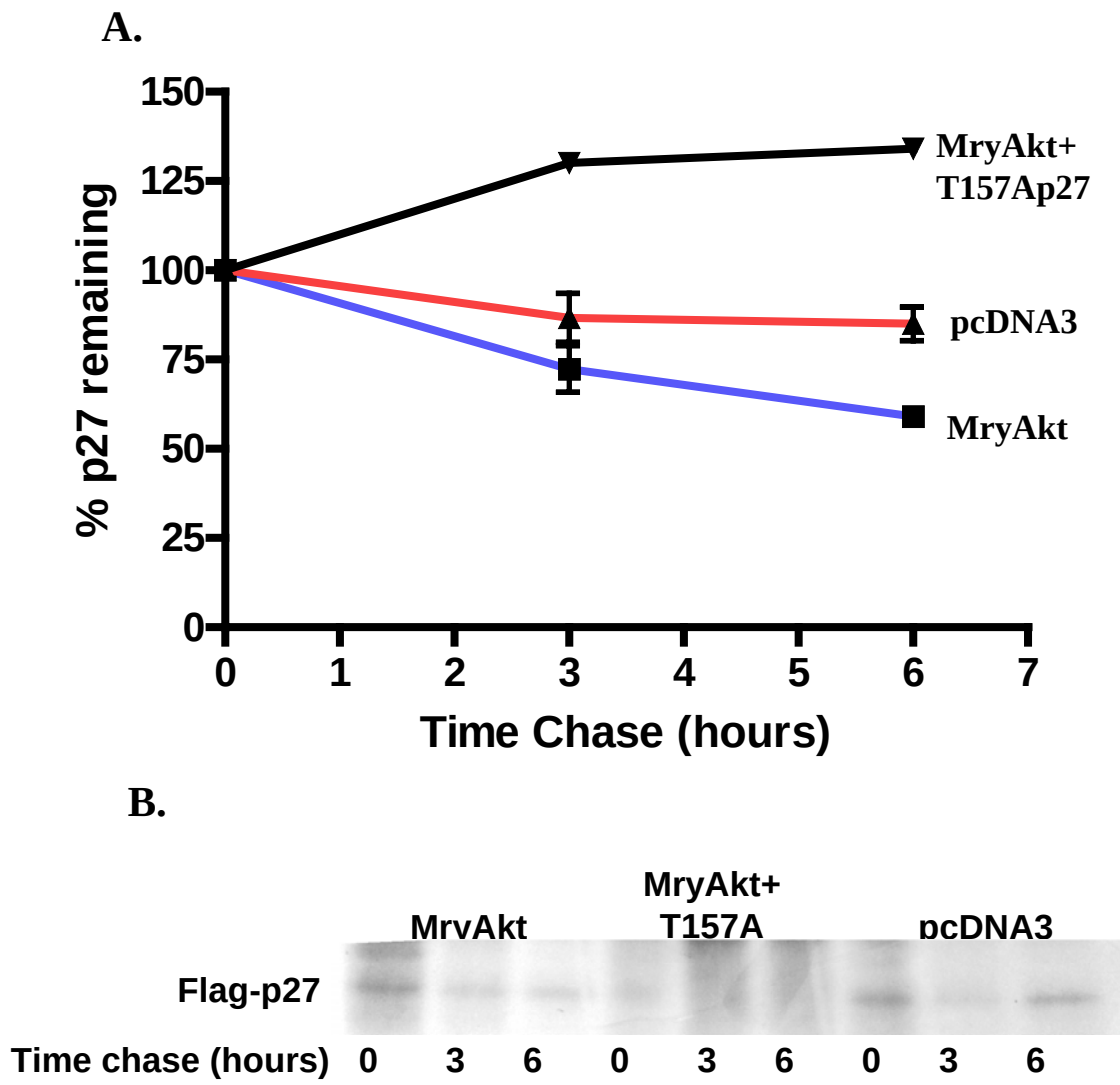


Figure 14: MryAkt does not stabilize p27 in the cytoplasm
(B) Cells were transfected with Flag-p27 or flag-T157Ap27 plasmid with pcDNA3 or MryAkt plasmids. At the indicated times, Cells were fractionated and flag-p27 was immunoprecipitated from cytoplasmic lysates. Immunoprecipitates were resolved by SDS-PAGE. (A) Quantitative results based on densitometry are presented as a graph: black =MryAkt+T157Ap27, red=pcDNA3+flag-p27, blue=MryAkt+flag-p27

p27 Degrades Similarly in the Nucleus in pcDNA3 and MryAkt Expressing Cells

We also wanted to investigate the role of activated Akt in the nuclear degradation of flag-p27. Cells were transfected with flag-p27 or T157Ap27 with MryAkt or pcDNA3. p27 degradation was analyzed by methionine labeling pulse chase experiments, and the cells were fractionated at three and six hours. Flag-p27 was immunoprecipitated from the nuclear compartment. Figure 15 shows that flag-p27 in pcDNA3 and MryAkt expressing cells degrades at a similar rate in the nucleus. T157Ap27 accumulates in the nucleus in MryAkt expressing cells, which agrees with data in figure 14A and figure 13A.

Estrogen Has a Similar Effect on p27 Stabilization in the Nucleus and Cytoplasm

Since estrogen can influence the localization of p27 through the Akt pathway (figure 10), it was important to investigate whether estrogen has any affect on stabilizing p27 in the separate cellular compartments through activating Akt. p27 degradation was analyzed by pulse chase analysis in cells transfected with flag-p27, T157Ap27 and dnAkt or pcDNA3, and all transfected cells were treated with estrogen during the chase. Flag-p27 was immunoprecipitated from the nuclear fraction (figure 16) or the cytoplasmic fraction (figure 17). Estrogen has no effect on the stabilization of p27 in control plasmid or dnAkt plasmid transfected cells in the nucleus or cytoplasm (figures 16 and 17). Cells transfected with T157Ap27 and treated with estrogen show a similar accumulation in both compartments (figure 16 and figure 17).

Mechanism of p27 Regulation by Activated Akt

Figure 18 demonstrates the potential mechanisms of p27 regulation by Akt found in this study. Akt is activated in cells either artificially or by transfecting cells with

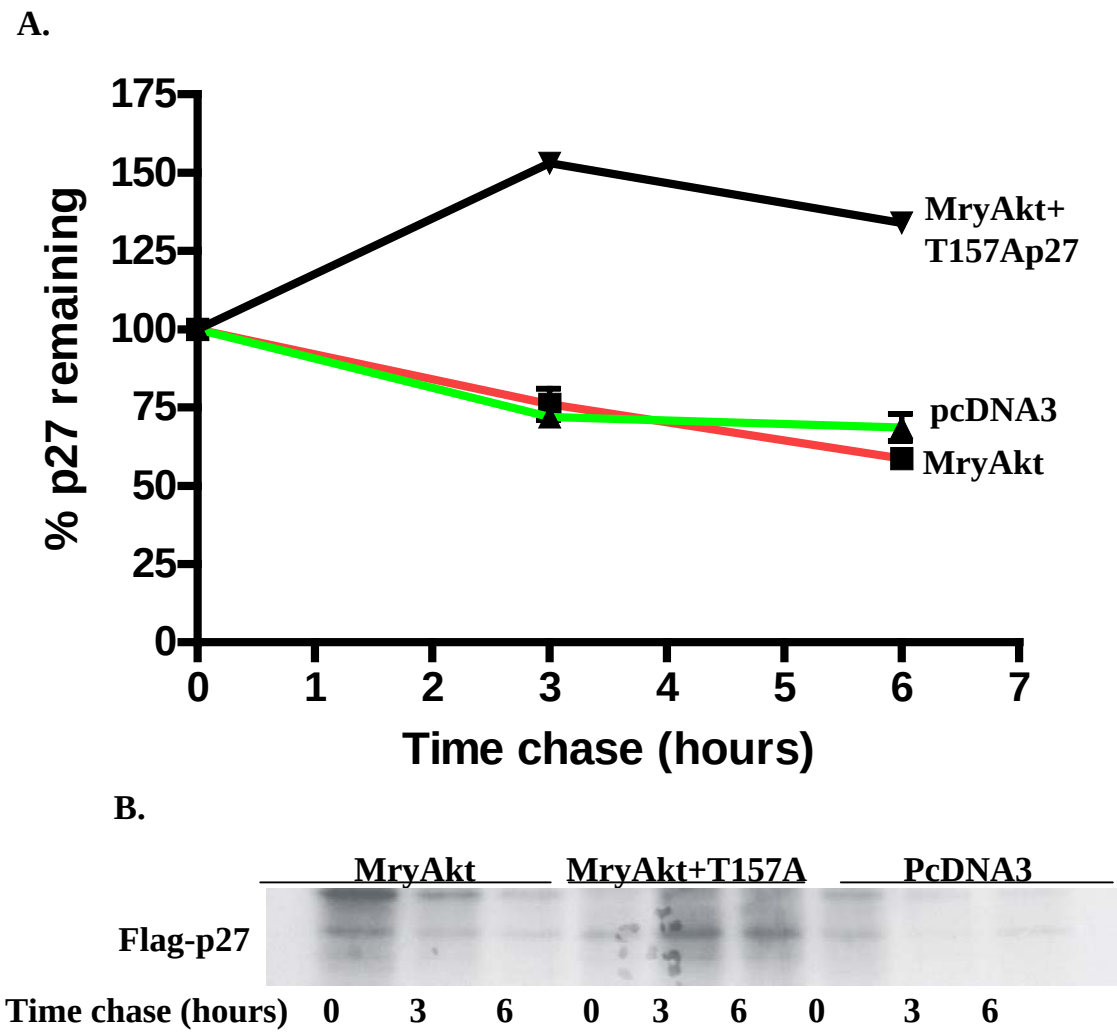


Figure 15 : MryAkt does not stabilize p27 in the nucleus
(B) Cells were transfected with flag-p27 or T157Ap27 plasmids and pcDNA3 or MryAkt plasmids. After 40 hours cells were labeled with ^{35}S methionine for 1.5 hours then chased with complete medium. Cells were collected at the indicated times and fractionated. Flag-p27 was immunoprecipitated from the nuclear lysate and resolved by SDS-PAGE. (A) quantitative results based on densitometry are represented as a graph.

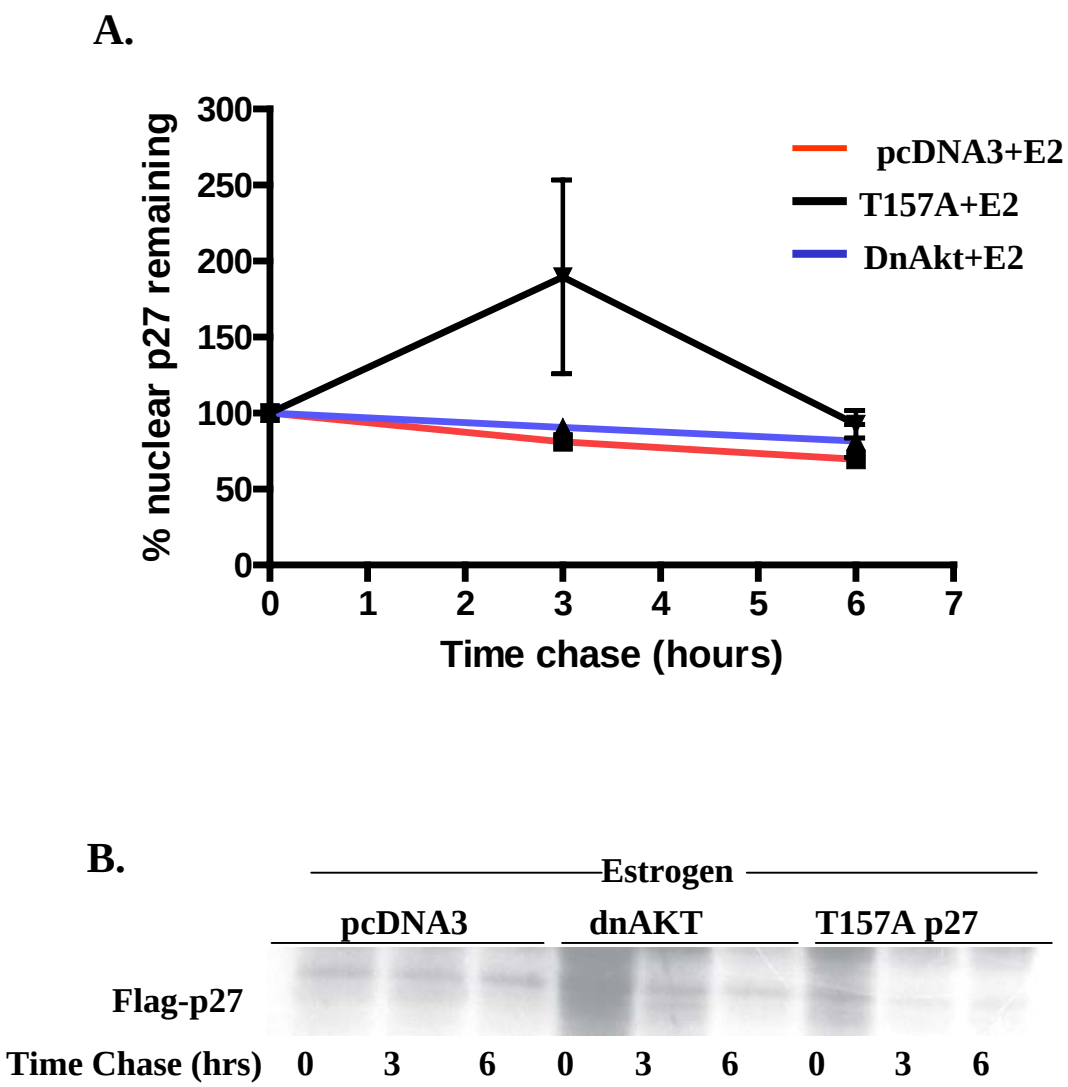


Figure 16: Estrogen does not stabilize p27 in the nucleus
(A) Cells were transfected with flag-p27 or T157Ap27 with the other indicated plasmids. Cells were labeled with ^{35}S methionine for 1.5 hours then chased for 3 and 6 hours in regular medium. Estrogen (E2) was added during chase to medium. Cells were collected, fractionated and p27 was immunoprecipitated from nuclear extracts and resolved by SDS-PAGE. (A) Quantitative results based on densitometry are represented as a graph.

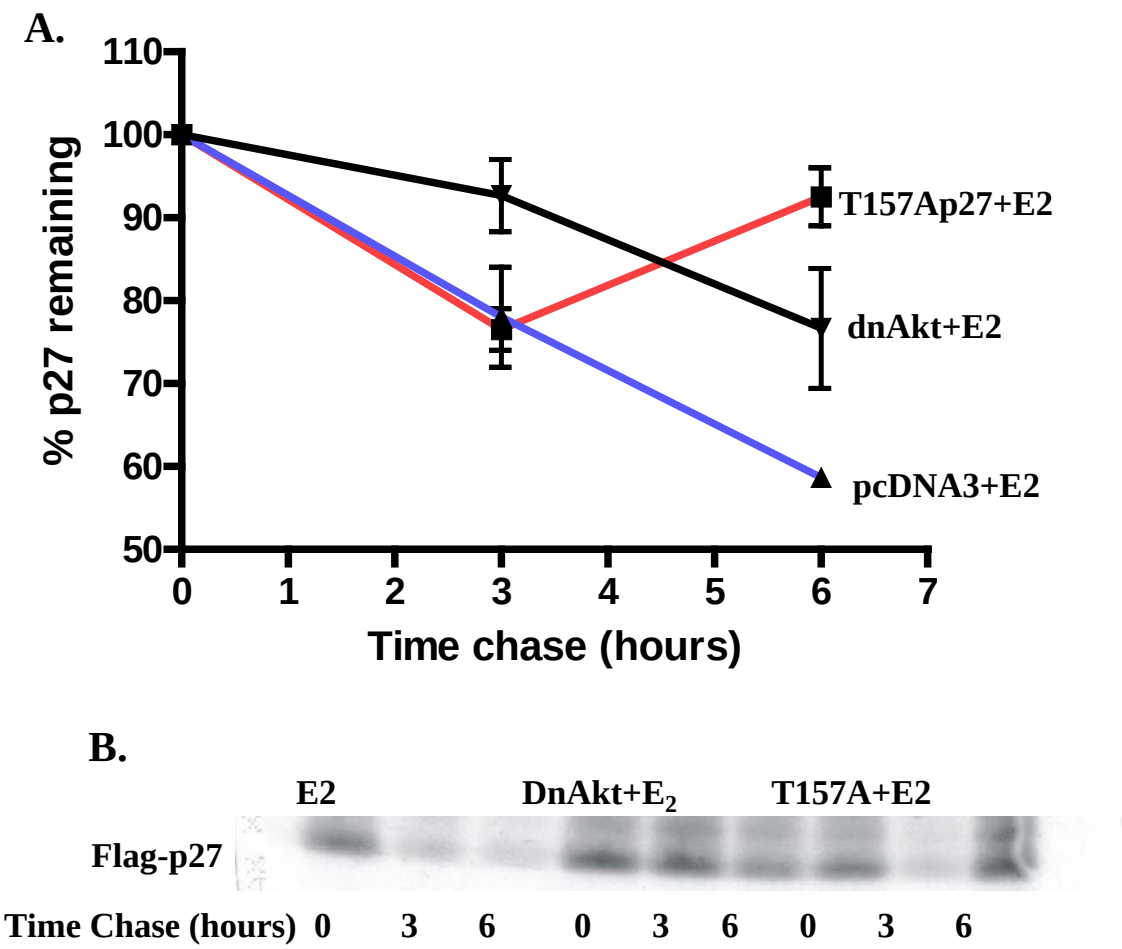
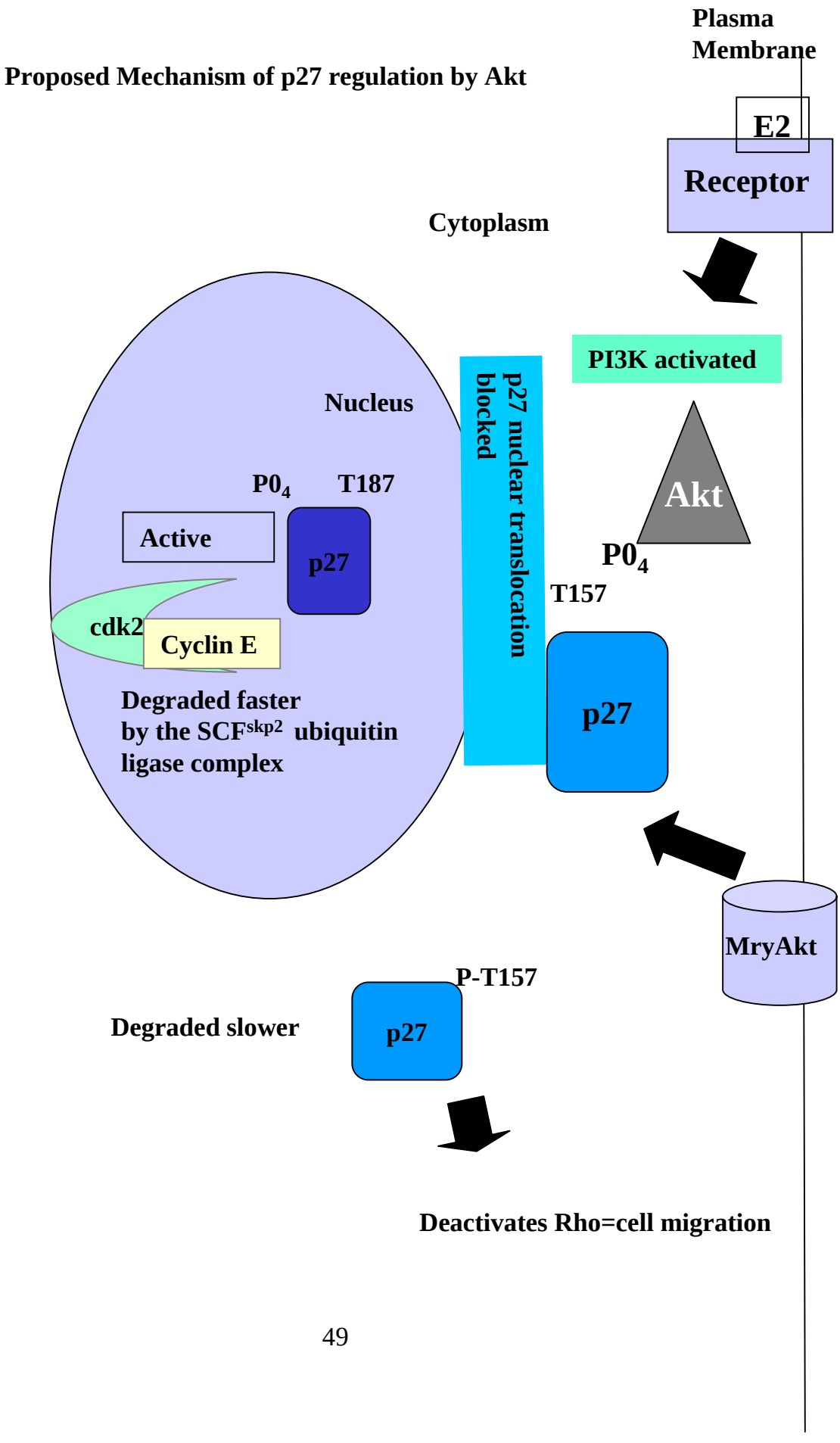


Figure 17: Estrogen does not stabilize p27 in the cytoplasm
(B) Cells were transfected with flag-p27 or T157Ap27 with one of the other indicated plasmids. Cells were labeled with ^{35}S methionine for 1.5 hours then chased for 3 and 6 hours in regular medium. Estrogen (E2) was added during chase to medium. Cells were collected, fractionated and p27 was immunoprecipitated from cytoplasmic extracts and analyzed by western blotting. (A) Quantitative results based on densitometry are represented as a graph.

Figure 18: Proposed mechanism of p27 regulation by Akt
Akt is activated by PI3 kinase, or artificially by MryAkt plasmid overexpression. PI3 kinase is activated by various receptors on the plasma membrane including estrogen (E2) receptors which bind E2 and activatemembrane receptors that activate PI3 kinase. When Akt is activated, it phosphorylates p27 on threonine 157 and this phosphorylation prevents p27 from entering the nucleus. p27 in the cytoplasm is more stable because degradation in the cytoplasm is slower. p27 may have cytoplasmic functions, like inactivation of Rho which leads to cell migration. In control cells were Akt in not active, p27 enters the nucleus and is degraded faster by the SCF^{skp2} protein ligase complex when it is phosphorylated on threonine 187 by the cyclinE/cdk2 complex.


Proposed Mechanism of p27 regulation by Akt
Plasma Membrane
E2
Receptor
Cytoplasm
PI3K activated
Akt
P0$_4$
T157
p27 nuclear translocation blocked
Nucleus
P0$_4$
T187
Active
p27
cdk2
Cyclin E
Degraded faster by the SCF^{skp2} ubiquitin ligase complex
p27
MryAkt
P-T157
Degraded slower
p27
Deactivates Rho=cell migration

MryAkt, or by treating with growth factors or mitogens (E2) that activate PI3 Kinase which activates Akt. When Akt is activated, it phosphorylates downstream targets like p27. When Akt phosphorylates nascent p27 on threonine 157, its nuclear translocation is prevented. Cytoplasmic p27 is more stable, due to the slower rate of degradation in the cytoplasm. Cytoplasmic p27 might have other functions, like the inactivation of Rho, which could increase cell migration. In control cells where Akt in not activated, p27 readily enters the nucleus where it can be phosphorylated (more quickly) by the SCF^{skp2} protein ligase complex, when it is phosphorylated on threonine 187 by cyclinE/cdk2 complex at G_1/S phase of the cell cycle.

V. DISCUSSION

Cdk inhibitors, like p27, are pertinent regulators of mammalian cell growth, and their deregulation has been linked to tumor growth and several cancers. This study was conducted to investigate the proteins and signal pathways that regulate the degradation or localization of p27. Recently the cytoplasmic retention of p27 has become of interest. p27 is retained in the cytoplasm in prostate, esophagus, thyroid, ovarian and breast carcinomas{7013}. This study suggests that Akt decreases p27 nuclear translocation by phosphorylating the protein on threonine 157. This mechanism was shown by both microscopic analysis of the cellular localization of p27, and by cellular fractionation. Previous studies have shown that cytoplasmic retention of p27 by active Akt increases Cdk2 activity. An active Cdk2/cyclin E complex initiates S phase of the cell cycle and eventual cell division. Cytoplasmic p27 may have other roles besides its nuclear function as a Cdk2 inhibitor. A recent study by Roberts et al (62), showed that cytoplasmic p27 has a role in cell migration by demonstrating that p27 null mice have decreased wound healing, due to suppressed migration of fibroblasts. GTPases of the Rho family play a large role in the regulation and remodeling of the cytoskeleton, a mechanism important in cell migration. According to the same study, p27 increases cell migration by the inactivation of Rho by reducing Rho-GTP levels. Cells lacking p27 have increased numbers of actin stress fibers and focal adhesions, which is indicative of Rho activation. Increases in cell motility and mobilization has been linked to tumor metastasis and vascularization. In order for tumor cells to spread throughout the body, mobilization of

the current tumor cells must occur. Also, since tumors grow at an accelerated rate and quickly deplete their supply of oxygen, they must vascularize in order to survive. If p27 plays a role in these processes, then p27's cytoplasmic retention by Akt might be conducive to tumor spread, and this could be an important target for future drug therapy. Also this might explain the growth promoting effects of p27 in heterozygote mice (4).

Active Akt in this study was also shown to influence newly synthesized p27. The synthesis of new p27 was determined with pulse experiments by labeling cells with ^{35}S methionine for the indicated times then collecting cells, and fractionating the nucleus from the cytoplasm. Over time, p27 protein in cells coexpressing MryAkt showed less nuclear p27. At three hours, the amount of p27 in the nucleus was similar in cells cotransfected with flag-p27 and either of the following: MryAkt, pcDNA3, dnAkt, or MryAkt and the p27 phosphorylation mutant T157A p27. By six hours, the amount of newly synthesized flag-p27 in the nucleus was less with active Akt present, and flag-p27 increased in the cytoplasm. This demonstrates a novel phenomenon that MryAkt does in fact influence new p27, and prevent its nuclear localization. PcDNA3 showed increasing amounts of p27 in the nucleus by six hours. DnAkt transfected cells expressing flag-p27 showed p27 increasing in the nucleus similar to pcDNA expressing cells, because Akt is inactive and not able to phosphorylate p27. T157A p27 behaves opposite to wild type flag-p27 in MryAkt transfected cells. T157Ap27 enters the nucleus and behaves similar to control (wild type p27 coexpressing pcDNA3).

In this study, estrogen also decreased the percentage of newly synthesized p27 in the nucleus by six hours. Estrogen is a potent mitogen for MCF-7 breast cancer cells (3),

and also has been shown to activate the PI3K/Akt pathway, and the Ras/Raf/Erk pathway (39,40,41). By activating Akt, estrogen could cause phosphorylation of p27 on threonine 157 and its subsequent cytoplasmic retention. This involvement of the estrogen activated Akt pathway is further supported by experiments in cells expressing dnAkt and treated with estrogen, which do not accumulate in the cytoplasm. Cells expressing T157Ap27 and treated with estrogen also behave similar to estrogen treated pcDNA and wildtype flag-p27 expressing cells. This result can be explained by studies showing that estrogen can cause rapid export of serine 10 phosphorylated p27 by the Ras/Raf/Mek/Erk pathway (33, 47).

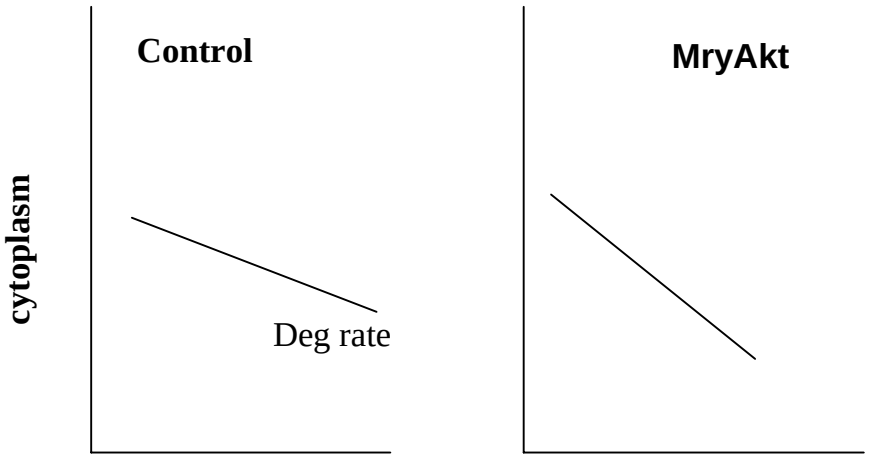
Decreased p27 levels associated with cancer are frequently caused by excessive proteolysis of the protein, rather than by allelic loss. Interestingly, in this study, active Akt, a known tumor promoter, was shown to increase p27 stability. This stabilization of p27 by Akt was supported by cyclohexamide experiments and ^{35}S methionine labeling pulse chase experiments. It was necessary to verify the cyclohexamide data with methionine labeling, because cyclohexamide halts all protein synthesis in the cell, therefore, the decreased production of other proteins could possibly influence the outcome of p27 degradation. Methionine labeling allows tracking of the protein of interest, while other proteins are synthesized normally, providing a more physiological environment for experimental analysis. The importance of the stabilization of p27 by Akt is still undefined. It simply might be an artifact of its cellular localization. Previously Ishida et al (29) showed that cytoplasmic p27 is degraded at a slower rate than nuclear p27, and by retaining p27 in the nucleus (by mutating it on serine 10) the half-life is

decreased. Conversely, this stabilization of p27 could be a result of active Akt; stabilization of a cell cycle inhibitor in response to increased growth signals could be the cells attempt at decreasing proliferation, rather than a direct effect of Akt on p27. Interestingly, the degradation of p27 in the nucleus was not different between cells coexpressing flag-p27 and MryAkt, or pcDNA3 (figure 15). The cytoplasmic degradation of flag-p27 in control cells was less then in cells expressing MryAkt and flag-p27 (figure14). This contrasts the results obtained with whole cell lysates, which showed repeatedly that MryAkt stabilized p27 protein levels. The only result in agreement with the whole cell lysate results is T157Ap27 and MryAkt coexpressing cells. These lysates showed a more stable T157Ap27 protein in the whole cell lysate, cytoplasmic, and nuclear fractions (figure13, 14, 15).

The discrepancy in degradation rates of flag-p27 following fractionation, and the whole cell lysate, is explained by the different distribution of p27 in the nucleus and cytoplasm in MryAkt transfected cells. Figure 19 shows the different percentages of p27 in control cells or cells with active Akt. Control cells contain more nuclear p27 than cells in which Akt is activated (figure 19B compare right and left panels). Cells that have active Akt possess more cytoplasmic p27 protein (figure 19A right panel). Therefore, in the whole cell lysate we are actually seeing the nuclear profile of p27 degradation in control cells, and the cytoplasmic profile of p27 degradation in MryAkt transfected cells (figure 19C).

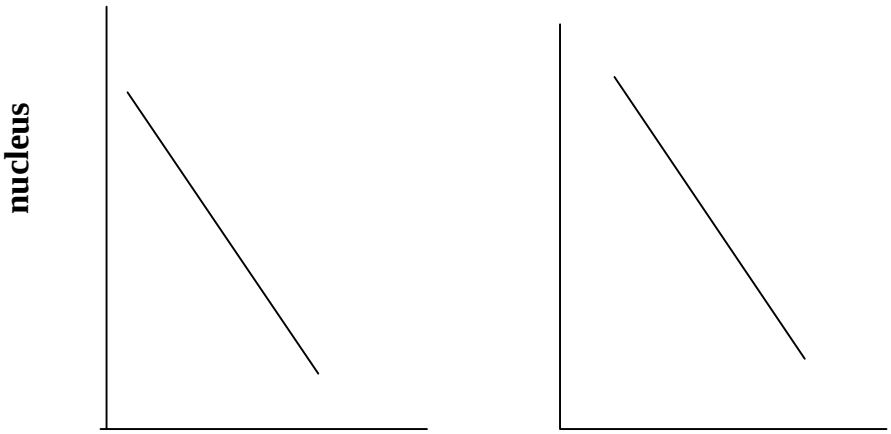
Figure 19: Distribution of p27 in control and MryAkt expressing cells (A) Proposed schematic of p27 in the cytoplasmic compartment in control (pcDNA3) and MryAkt expressing cells. Sloping line indicates the rate of p27 degradation over time. % p27 protein indicates the percentage of total p27 in that cellular compartment as a result of treatment(B) Graphic representation of the proposed distribution (represented as a percentage) of p27 in the nuclear compartment in control and MryAkt expressing cells. (C) Graph of the distribution of p27 and it's rate of degradation in the whole cell lysate in control and MryAkt expressing cells

A.



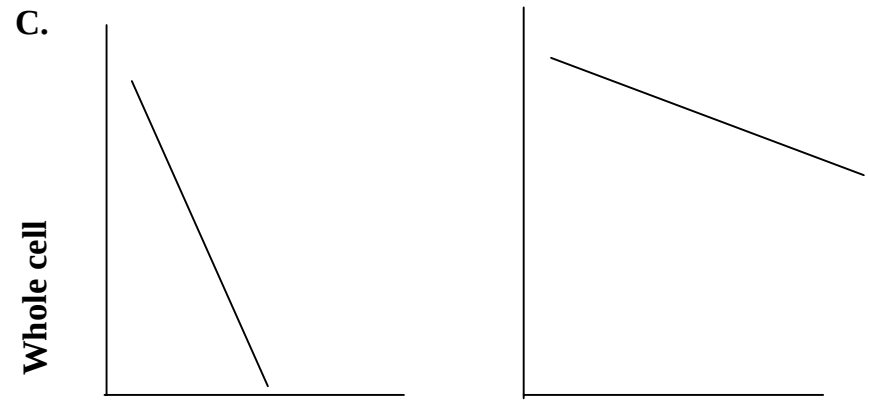
% p27 protein **30** **70**

B.



% p27 protein **70** **30**

C.



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VITA

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